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13. ABSTRACT *(Maximum 200 Words)*

This project aimed to identify new genes that connect TGFβ with cell cycle control. Most breast cancers have altered responses to TGFβ and learning more about how TGFβ controls cell cycle progression is an important avenue for future therapies. Using *C. elegans* as a model system, we have induced cell cycle arrest using mutations in TGFβ that promote dauer formation. Using a *rnr::gfp* reporter for cell cycle progression, we have undertaken a genetic screen to find mutations that alleviate the cell cycle repression. In complementary studies, we have used DNA microarrays to profile mRNAs from animals entering the TGFβ-induced dauer phase. Analysis of this data indicates that many known cell cycle regulators have altered expression profiles, as expected. We are mining the microarray data for new and novel genes whose expression profiles mimic those of the known cell cycle regulators. One gene that was found to be down regulated in these experimental conditions is a homolog of *down regulated in metastasis* (DRIM). DRIM was originally discovered as a gene whose expression changes in breast cancer cells that metastasis to lung cancers. Our data shows that DRIM is a likely downstream target of TGFβ signaling and may be necessary to carry out its growth regulatory effects. Further studies will be necessary to explore DRIM as a possible therapeutic agent.

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Table of Contents

INTRODUCTION

More than 80% of breast cancers are derived from epithelial cells. A potentially fruitful avenue toward controlling breast cancers is to learn how to control the growth of epithelial cells. One of the most potent inhibitors of epithelial cell growth is TGFβ. TGFβ signaling plays a major role in the normal development of the breast and in the progression of breast cancers by controlling exit from the cell cycle. Thus, the regulation of cell cycle exit constitutes a promising avenue towards treating breast cancers (Catzavelos *et al.*, 1997).

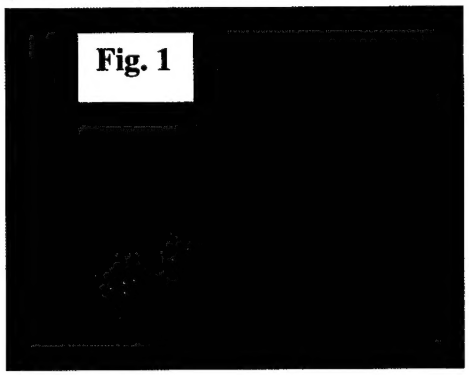
Our approach. We are utilizing two complementary approaches in *C. elegans* to find cell cycle regulatory genes that respond to TGFβ signaling. First, in genetic screens using a cell cycle reporter gene, *ribonucleotide reductase*, we will look for mutations in loci that release dauer animals (a TGFβ induced developmental stage) from their cell cycle arrest (Hong *et al.*, 1998). Many of these genes may be directly regulated by the TGFβ pathway. Secondly, we are taking a complementary molecular approach to find genes regulated by the *C. elegans* TGFβ pathway. Using RNA from arrested animals and from animals released from dauer arrest, we have probed DNA microarrays containing the 17,700 genes (of 19.000 genes known) of *C. elegans* to identify ones whose expression is altered as animals are released from TGFβ induced arrest. These two approaches should provide us with candidate genes that connect the TGFβ pathway with specific novel, regulators of cell cycle arrest. To extend our findings, in future experiments we will validate our results by examining the effects of the vertebrate homologs on control of cell cycle progression in cell culture assays. This information will increase our understanding of how cell cycle regulation is achieved, how TGFβ regulates these events, and will provide reagents for the design of novel therapeutics.

BODY

***Task #1*. Generate strains for genetic screens and do a pilot screen to verify efficacy of screen.** In order to carry out our genetic screen, we need a reporter for cell cycle progression. Since *C. elegans* animals are transparent, the green fluorescent protein (*gfp*) can be detected *in vivo* using a simple dissecting microscope. At the beginning of this granting period, a reporter for ribonucleotide reductase was available (*rnr::gfp)*. However, it showed an odd genetic property—it acted as a recessive rather than a dominant gene and gave low levels of expression. To overcome these problems, we designed two new constructs. First, we duplicated the promoter region of the *C. elegans* ribonucleotide reductase

gene (*rnr*) and inserted it into an appropriate vector for transformation into animals. The rationale for duplicating the promoter region is to increase expression levels. Since most constructs of this type act as dominant alleles, we felt that a new construct would likely solve the problem of it acting in a recessive manner.

Generation of a new transgenic line. We examined the expression levels of constructs containing two or three duplicated *rnr* promoter regions to find the ones that were most intense. One line, containing two copies of the promoter gave the best results. It was chosen for further characterization. It was integrated into the worm genome so that all the cells in the animals contain the construct, thereby preventing loss in some tissues during development. We used UV light to integrate the construct into the worm genome (see UV Integration protocol in appendix). Several technical problems were encountered with the integration, primarily affecting the frequency of integration. These are standard laboratory techniques, but somewhat new to our lab and it took some additional effort to resolve. However, we did obtain two independent, integrated lines (Fig. 1). These lines were then assessed for their expression levels. Both lines give similar expression levels and one was chosen for use in our genetic screen.



A daf-4 allele was recombined onto our reporter strain. Mutations in a TGFβ pathway in *C. elegans* (the dauer pathway) results in cell cycle arrest at the L2 larval stage. Therefore, we need to incorporate dauer mutations in our *rnr:gfp* strains for our genetic screen so that they will show TGFβ-induced cell cycle arrest. We chose *daf-7* and *daf-4* mutations (null alleles of the ligand and receptor, respectively), since they completely induce dauer formation. Two independent strains have been constructed. We will begin our screen with the *daf-4* strain, since this *daf-4* allele was used in our microarray experiments described below.

An alternative reporter construct was generated. We have generated an alternative construct for detecting cell cycle progression, a *PCNA::gfp* construct. The *PCNA* gene is highly conserved in *C. elegans* and serves as another excellent reporter in vertebrate systems. The same strategy of duplicating the promoter regions was used to increase its expression. It will be used as a secondary marker to eliminate the rare mutations which affect only the promoter of *rnr*. These two strains are available to the research.

***Task #2, #3, #4* (these tasks are intertwined and are discussed together). Perform the genetic screen, characterize the mutants, and clone interesting loci.**

A pilot genetic screen was performed using our new strains. As described above, we characterized an existing *rnr::gfp* strain, which proved to be unsatisfactory, prompting us to re-engineer the strain. Once we had re-engineered the construct, injected it into nematodes, integrated it into the genome, we were then able to use it in a pilot screen to test its efficacy. At the L2 stage, in animals containing the *daf-4* mutation and the *rnr::gfp* reporter, no gfp was observed, suggesting that the construct was not leaky and would not interfere with our screening protocol. A pilot screen allowed us to examine some of the screening parameters (ie. how many genomes can we process per two week period, how reliable is the *gfp* reporter, how many false positives do we get, *etc.*). This enabled us to evaluate the ease of scoring mutants under a compound microscope (more laborious) vs. a dissecting microscope and to evaluate throughput. For our pilot screen, we examined about 1000 genomes for mutations affecting cell cycle arrest in dauer animals. We fine tuned the protocols and are poised to continue the genetic screen.

Preparations for the screen took longer than we had originally hoped, but the good news is that the screen is robust and should allow us to identify many interesting mutants. A schematic of the genetic screen is shown in the appendix (see Genetic screen scheme). In the course of our work using nematodes, we have gained extensive experience on characterizing and mapping mutants, particularly using SNP mapping. SNP mapping has become the method of choice to map mutants to small physical regions of the chromosome, as a prelude to cloning. These techniques require a significant investment of time, and our prior experience will be valuable in mapping mutants from this screen.

***Task #5.* Do differential hybridizations with DNA microarrays.** As a complementary approach to our genetic screens, our second major aim was to take a molecular approach to find cell cycle genes regulated by the *C. elegans* TGFβ pathway. This task seeks to identify genes that are regulated by a TGFβ-induced dauer state. RNA is made from animals just entering the dauer stage and compared to RNA made from non-dauer animals. This RNA is used as a probe to DNA microarrays to determine which genes are induced or repressed. Since the dauer state is a TGFβ induced state, it should allow identification of those genes that connect TGFβ with cell cycle regulation.

Collection of RNA from staged animals. When animals are just beginning to enter the dauer stage, pharyngeal pumping changes. This can be used to precisely stage the animals for RNA collection. Using this phenotypic change as a signal, we collected and froze animals that were previously synchronized to use as a source of RNA. Animals were synchronized by bleaching gravid hermaphrodites, which releases synchronized eggs. These eggs were allowed to hatch and were grown under appropriate conditions. Total RNA was made from pools of animals (see RNA protocol in appendix). Briefly, polyA RNA was made from total RNA using an Invitrogen FastTract 2.0 mRNA kit. Three independent sets of RNA were generated using these protocols.

Hybridization to microarrays. RNA was sent to the Microarray Facility at Stanford University. Dr. Stuart Kim, at Stanford University, operates a free microarray facility for *C. elegans* researchers (funded by NIH). His microarrays contain about 17,700 *C. elegans* genes (of 19,000 total). These were probed with our labeled mRNA, and the results were sent to us for analysis (see Appendix). In the last few months of the grant, Affymetrix began selling microarray chips containing *C. elegans* genes, but these were not available during the time we carried out these experiments.

Suggestions for improvements to the microarray protocols. Commonly, investigators perform microarray experiments in triplicate. This allows one to develop a reasonable statistical base to determine if results of two different experiments differ statistically. However, the robustness of the finding increases as one adds more replicas to the experiments. Although there are still ongoing debates about how many replicas to include, one to two more could provide more statistical significance and a great number of genes that fit our criteria. If more replicas are done, then many genes whose statistics are borderline, might move into the highly significant category, thereby increasing the numbers of genes that show a change.

Mining the microarray data. The techniques used to analyze microarray data is new for most of us in the field and we are in the process of learning how to mine the data in a sophisticated manner. One chooses a statistically significant level of RNA expression change. Then, genes are grouped according to increases or decreases in mRNA levels. This results in two groups of genes—those that increase in expression and those that decrease in relation to TGFβ function.

The first step in analyzing the data is to generate an average and standard deviation of the signal from the replicas of each gene. Then a t-

test is used and a P test to determine if the control and experimental values are different. We are using a 95% and 99% confidence levels to select the genes which show the most change. This is around a 2-fold change in expression levels. At the 95% level we find that 104 genes are upregulated and 504 are down-regulated. At the 99% confidence level, there are 94 genes whose expression levels change.

Cluster analysis. Once we had a statistically meaningful data set, we used clustering software that identifies groups of genes, or clusters, that share similar expression profiles. Genes that cluster may be involved in similar biological functions, even if their biochemical functions are distinct. For example, there may be genes that are turned up after entering the dauer stage, and track the expression profiles of cell cycle genes. We have begun making these clusters and grouping genes based upon similar expression profiles.

One of the main goals is to identify new genes that connect TGFβ to cell cycle regulation. To evaluate the robustness of our data, we examined the status of known cell cycle regulators from these experiments. Since the animals we selected to obtain mRNA from are just entering dauer, we expect that their cell cycle machinery should be turned down or off. As expected, we find that some cell cycle genes are more highly expressed, while others are reduced in expression. Most importantly, we find that cyclin D and cyclin E are turned down in these experiments (see Appendix). This independently confirms that we chose the correct timepoints/animals for analysis. Now we are examining our microarray data to find novel genes whose expression levels change as a result of TGFβ function and track the known cell cycle genes.

The expression of DRIM changes in TGFβ mutants. One exciting result has been obtained from our microarray work. This discovery was made possible by the combination of different pieces of the puzzle from several projects, including this work. Using an activated TGFβ receptor (*thick veins*) expressed in Drosophila wings, we performed a genetic screen to find modifiers of TGFβ. Twenty modifiers were found and mapped to the genome. Using a hypomorphic allele of *thick veins*, the Hoffmann group (UW-Madison) did a similar genetic screen, and identified one new locus that modifies TGFβ. We complement tested our mutations to his and found that both screens had uncovered the same locus, further bolstering the idea that this mutation was a *bona fide* modifier of TGFβ. Cloning of the gene revealed it corresponded to *down regulated in metastasis* (DRIM)(M. Hoffmann, personal communication). DRIM was originally discovered in a differential display experiment that sought

differences between breast cancer cells and a metastatic lung tumor derived from breast cancers (Schwirzke et a., 1998). This gene is found in many different phyla and is novel, so there are no clues as to its biochemical function. The original cell line experiments offered no clues to the signaling pathways or growth control pathways it was involved in. However, our Drosophila data shows it is necessary for the TGFβ pathway to carry out its growth control. This is our first clue as to the role of DRIM in growth control and has been shown to suppress the activated TGFβ pathway in *Drosophila.* This afforded us an opportunity to see if the regulation of this gene might be altered when TGFβ was mutant in our *C. elegans* experiments.

DRIM is a novel protein. DRIM is a large novel protein of about 2700 amino acids that contains several HEAT (huntingtin-elongation-A subunit-TOR) repeats scattered throughout the protein (Schwirzke *et al.*, 1998). These motifs are found in the huntingtin protein (Gusella and MacDonald, 1998), the PR65/A subunit of protein phosphatase 2 (Groves *et al.*, 2001), and number of other proteins. An unequivocal role for the HEAT in promoting protein-protein interactions has been established for some of these HEAT-containing proteins (Groves *et al.*, 2001). DRIM also contains a putative nuclear localization domain, and a leucine zipper. This suggests that it might be involved in transcription, but that has not been established.

Does C. elegans have a homolog of DRIM? Searches of the database reveals a homolog of about 22% identity and 42% similarity to the human protein throughout the length of the protein. We wondered if DRIM was regulated by the TGFβ pathway or acted in a parallel fashion to augment TGFβ signaling. Therefore, we examined our microarray data. DRIM was found to be a downstream target gene of TGFβ in *C. elegans*, whose regulation responds to TGFβ levels. Checking its expression profiles in other experiments was instrumental. It is also up-regulated in *Drosophila* ovaries which over-express *decapentaplegic*, a TGFβ ligand, and is virtually off in *decapentaplegic* mutant ovaries (T. Xie, Stower's Institute, pers. comm.), consistent with our microarray data in *C. elegans*. This data is consistent with the hypothesis that DRIM is a new downstream target of TGFβ and is required for some of its regulatory growth properties. This makes it an ideal candidate for further study. This is exactly the type of gene that we hoped to find in this project—a new regulator of TGFβ growth control that is implicated in breast cancer.

Task #6. Clone mammalian homologs. One of our tasks was to clone the mammalian homologs of genes we identify in our *C. elegans*

experiments. Through the experiments outlined above, DRIM is one of the genes whose expression profiles change in TGFβ mutants, and has a strong human homolog. As the analysis of the other genes in our dataset proceeds, we will focus on those that have vertebrate homologs. Currently, about 45% of the genes whose expression changes in our experiments have vertebrate homologs, so finding human counterparts to study will be relatively easy.

KEY RESEARCH ACCOMPLISHMENTS

1) integrated new *rnr::gfp* constructs into the nematode genome using UV light
2) crossed appropriate genetic markers (*daf-7* and *daf-4*) into integrated nematode strains to prepare for genetic screens
3) performed a pilot screen to test efficacy of screening protocol
4) completed mRNA preparation from dauered animals in triplicate
4) sent mRNA to microarray facility and obtained microarray data from our experiments
5) found many unknown genes whose expression profiles mimic those of known cell cycle regulators
6) found that DRIM is down regulated in dauered animals, as it is in metastatic breast cancer cell lines, and is likely to be necessary for TGFβ's growth control

REPORTABLE OUTCOMES

Grants applied for based upon this work.

A proposal was submitted to the DOD Army Breast Cancer Program (June, 2002) to continue the analysis of genes from this study, with a special emphasis on the study of the DRIM gene.

A Program Project Grant to the NIH is being written with members of the Cancer Institute in New Jersey to further study the connection between TGFb and breast cancer (Pis: Drs. Michael Reiss, Richard Padgett, Fang Liu, and Garth Patterson), expected submission, February, 2003.

Review Articles.

1. Das, P., L.L. Maduzia, and R. W. Padgett (1999) Genetic Approaches to TGFβ Signaling Pathw*ays,* **Cyto. and Growth Factor Reviews** 10:179-186.

2. Patterson, G. I. and R.W. Padgett (2000) TGFβ-related Pathways: Roles in *C. elegans* Development, **Trends in Genetics** 16:27-33.

3. Zimmerman, C. and R.W. Padgett (2000) TGFβ Signaling Mediators and Modulators, **Gene** 249:17-30.

Abstracts for meetings.

Roberts, A., S-L. Wu, C. Zimmerman, and R.W. Padgett (2001). TGFβ Signaling and Gene Expression, *International C. elegans Meeting, June, 2001, Los Angeles, CA*.

Roberts, A., S-L. Wu, K. Checchio, and R W. Padgett (2002). Gene Regulation by TGFb Signaling In *C. elegans, East Coast International Worm Meeting, June, 2002, Durham, NH.*

Invited talks.

Signal Transduction Pathways and Regulation of Gene Expression as Therapeutic Targets, Luxembourg, January, 2000.
University of Umea, Umea, Sweden, January, 2000.
Ludwig Institute, Uppsala, Sweden, February, 2000.
CABM Symposia, Rutgers University, October, 2000.
Keystone Symposia, Integration of Signaling Pathways in Development, Keystone, CO, January, 2001.
FASEB Summer Research Conference, The TGFβ Superfamily: Signaling and Development, Tucson, AZ, July, 2001
University of North Carolina-Chapel Hill, November, 2001
Queens College, CUNY, May, 2002

Poster Presentations.

Zimmerman, C.M., L.L. Maduzia, A. F. Roberts, C. Savage-Dunn, and R. W. Padgett, Defining New Components of TGFβ Signaling Pathways in *C. elegans*, poster presentation at the Salk Oncogenesis Meeting, La Jolla, CA, August, 2001.

Lisa L. Maduzia, Pradnya Shetgiri, Srikant Krishna, Cathy Savage-Dunn, Huang Wang and Richard W. Padgett, *lon-1*, a putative downstream target of TGFβ signaling, *International C. elegans Meeting, June, 2001, Los Angeles, CA*

Personnel involved with this project.

Shi-Lan Wu, postdoctoral fellow
Tina Gumienny, postdoctoral fellow
Huang Wang, research technician
Andrew Roberts, graudate student
Cole Zimmerman, postdoctoral fellow
Kristin Checchio, undergraduate honors student

CONCLUSIONS

During the granting period of this DOD Idea Grant, we have made substantial progress toward our original goals. We had two major aims—to generate the necessary strains to carry out a genetic screen in *C. elegans* that will allow us to find genes that connect TGFβ to cell cycle regulation, and to generate microarray data from *C. elegans* animals that are undergoing TGFβ-induced cell cycle arrest. These experimental approaches were technically very successful and resulted in the identification of DRIM, a gene down regulated in metastasis that was not previously associated with TGFβ.

We have successfully made integrants of our newly improved reporter construct and established appropriate strains for conducting a genetic screen. A pilot genetic screen showed that the reporter is functioning as expected and that this screen should be able to identify new targets for TGFβ-induced cell cycle arrest.

We have successfully obtained microarray data from the *C. elegans* facility and are in the process of mining the data. We find that known indicators of cell cycle progression are transcriptionally reduced in our mRNA samples, supporting the idea that we picked appro-priately aged animals, and that the approach works. One gene that appeared in our microarray data was DRIM, a gene that we have been able to show is a target of TGFβ signaling and is linked to metastasis of breast cancer. Our results link it to TGFβ and show that it is a downstream target of TGFβ. As we learn more about DRIM and the other genes that tract its expression profile, some of these genes may become useful targets for therapies.

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Groves M.R., N. Hanlon, P. Turowski, B.A. Hemmings, and D. Barford (1999). The structure of the protein phosphatase 2A PR65/A subunit reveals the conformation of its 15 tandemly repeated HEAT motifs, Cell 96:99-110.

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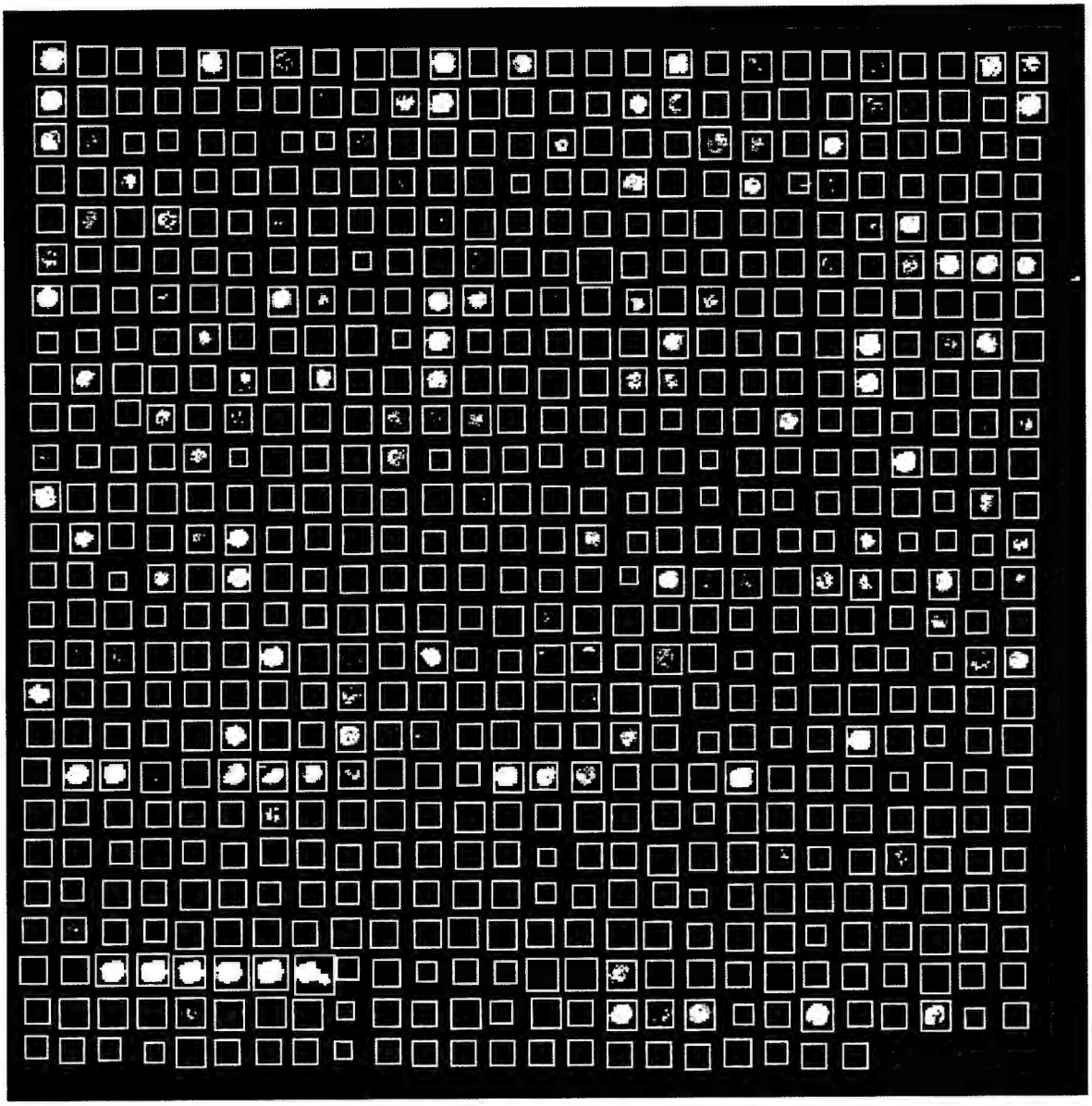


RNA Isolation for Microarrays

N2 versus DR1359 (genotype: daf-4 (m592))

Collecting worms

1. Maintain worms on 15cm NGM/agar plates at a density of about 20, 000 worms/plate at 15^{0}C. Egg prep. at each generation to keep worms clean and synchronous.
2. Collect 500,000 or more gravid animals from each of the two strains and egg prep. Resuspend eggs at about 100,000 worms/ml in sterile M9 and hatch at 15^{0}C overnight in a flask with gentle rocking.
3. Count hatched L1's to be sure there are at least 3,000,000 total for each strain. Spin down L1's and resuspend in fresh M9 at about 200,000 worms/ml. Plate according to the following schedule:

9:00am: plate daf-4 strain sample plate (1.5 cm NGM agar plate - 10.000 L1s)
11:00am: plate daf-4 strain big plates (15 cm NGM agar plates - 100, 000 L1s/plate)
11:00am: plate N2 sample plate (same as above)
1:00pm: plate N2 big plates (same as above)
Incubate all of above at 25^{0}C.

4. The following morning begin monitoring pumping on both sample plates and big plates on an hourly basis. Harvest N2 big plates when sample plate worms are in L2/L3 molt (when % non-pumpers no longer decreases). Harvest daf-4 big plates when % non-pumpers on the sample plate reaches 30-50.
5. Use DEPC treated M9 to rinse plates at least three times to collect L2s. Wash worms a couple of times with M9 and then resuspend in 4x volume of Trizol reagent. Freeze in liquid N_2, thaw at 37^{0}C and freeze again in N_2, thaw, freeze, and store at -80^{0}C.

Total RNA Prep:

Following the protocol from Dr Kim's lab.

mRNA Prep:

Using Invitrogen FastTrack 2.0 Kit

10/10/00
N2: 0.76 ug/ul
daf-4: 0.69 ug/ul
11/15/00
N2: 1.46 ug/ul
daf-4: 0.45 ug/ul
12/20/00
N2 1.12 ug/ul
daf-4: 1.36 ug/ul

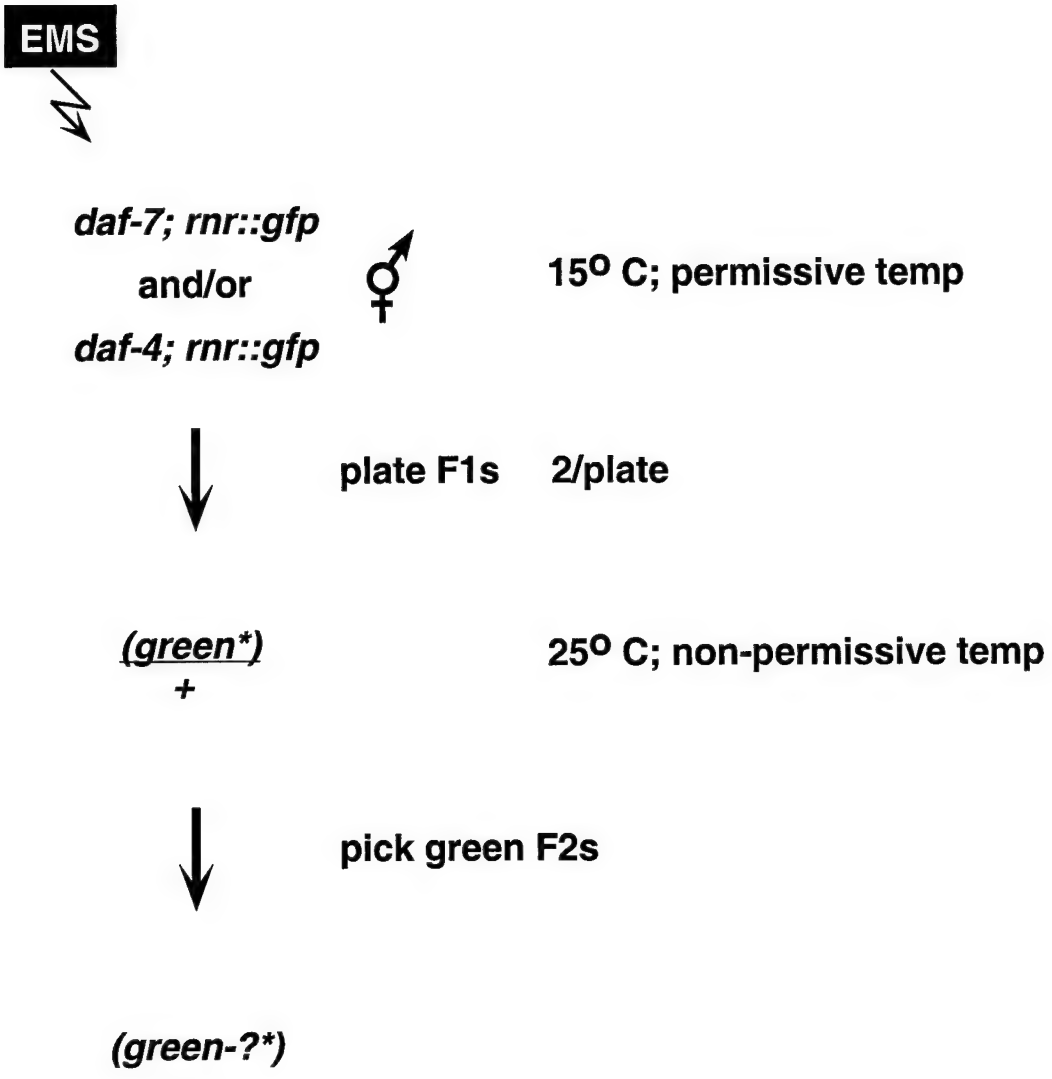
Gene name	directional agreement	upregulated in	Description
F35G12.9	y	?	Protein containing a C3HC4-type (RING finger) zinc finger domain, has similarity to S. cerevisiae cell cycle proteins Apc11p and Hrt1p
C06A1.1	y	Daf-4	Member of the proteasome complex protein family
C07G1.3	y	Daf-4	Serine/threonine protein kinase of the PCTAIRE subfamily of protein kinases, all of which are structurally related to p34cdc2 cyclin-dependent protein kinase
C13G3.3A'	y	Daf-4	Putative B' regulatory subunit of protein serine/threonine phosphatase 2A (PP2A)
C41C4.8	y	Daf-4	Member of the proteasome complex protein family
D2045.6	y	Daf-4	Cell cycle control protein, member of the cullin family of cell cycle control proteins
F43D2.1	y	Daf-4	Putative cyclin, protein with strong similarity to human CCNK protein, a cyclin K
F44B9.3	y	Daf-4	similarity to human CCNT1, cyclin T1
F46A9.5	y	Daf-4	Putative cyclin A/CDK2-associated transcription elongation factor, has strong similarity to H. sapiens TCEB1L gene product
F55B12.3	y	Daf-4	Protein which negatively influences LIN-12 signaling; binds the presenilin homolog SEL-12
R07E4.6	y	Daf-4	Regulatory subunit of cAMP-dependent protein kinase that binds KAP-1
R08C7.2	y	Daf-4	Member of the Cdc50p-like protein family
T05A6.1	y	Daf-4	G1-phase cyclin-dependent kinase inhibitor; member of the CIP/KIP (p21/p27) family of cyclin-dependent kinase inhibitors
T27E9.3	y	Daf-4	Serine/threonine protein kinase with similarity to cyclin-dependent protein kinases
T28B4.1	y	Daf-4	Protein containing an F-box domain, has weak similarity to S. cerevisiae Grr1p, an F-box protein involved in SCF/ubiquitin-mediated degredation of cell cycle regulator
B0261.2	y	N2	Member of the phosphatidylinositol kinase protein family
C37A2.4	y	N2	Putative cyclin E2, has strong similarity to human and D. melanogaster cyclin E proteins, has similarity to S. cerevisiae Clb proteins
C43E11.1(	y	N2	strong similarity to Human CDC18L, cell division cycle 18
C52D10.8	y	N2	similarity to the SKP1 family of proteins, putative paralog of C. elegans C52D10.6 and Y47D7A_138.D
F10B5.6	y	N2	similarity to S. cerevisiae Cdc16p, a component of anaphase-promoting complex (APC) that is required for cyclin degradation and for the metaphase-anaphase transit
F18C5.3	y	N2	strong similarity to human DRIM, which is down-regulated in metastasis
F29B9.6	y	N2	Member of the ubiquitin-conjugating protein family
F32A11.2	y	N2	similarity to S. pombe and human RAD17, cell cycle checkpoint proteins
F32E10.4	y	N2	Member of the karyopherin-alpha protein family
F34D10.2	y	N2	strong similarity at the N-terminal half to human CDC45L protein, cell division cycle 45-like protein
F44B9.4	y	N2	strong similarity to human CCNT1, cyclin T1
F45E12.3	y	N2	Member of the cullin family of cell cycle control proteins
F48E8.7	y	N2	strong similarity to human Hs.159269, a cyclin A/CDK2-associated p45 (Skp2) protein
F53G2.7	y	N2	strong similarity to human MNAT1 (menage a trois 1) a putative Ring finger containing protein, a CDK7-cyclin H complex assembly factor
F54H12.5	y	N2	Member of the F-box domain protein family
F59H6.7	y	N2	similarity to cyclins of human, D. melanogaster and S. cerevisiae, putative paralog of C. elegans F08F1.9
K06H7.5	y	N2	Putative cell cycle control protein, contains a cullin domain, has weak similarity to S. cerevisiae anaphase-promoting complex component APC2, has weak similarity to
K08E7.7	y	N2	Member of the cullin protein family of cell cycle control proteins
M03F8.3	y	N2	Putative ortholog of Drosophila crooked neck (crn) and S. cerevisiae Syf3p, putative pre-mRNA splicing factor
R03D7.7	y	N2	Protein that functions in development of the germ lineage, has similarity to D. melanogaster nanos
R10E4.4	y	N2	Member of the MCM initiator complex (DNA replication) protein family
T05G5.3	y	N2	Serine/threonine protein kinase, putative ortholog of human CDC2 and S. cerevisiae Cdc28p cyclin-dependent serine/threonine protein kinases involved in cell-cycle r
T06E4.3	y	N2	Putative ortholog of H. sapiens ATR protein (ataxia telangiectasia and Rad3 related) FRAP-related protein
T06E6.2	y	N2	Member of the cyclin B protein family
Y38F1A.5	y	N2	Probably a Cyclin D homolog, may act to control postembryonic G1 progression
Y43E12A.1	y	N2	Member of the cyclin B protein family

Y49F6B.R	y	N2	Putative cyclin H
Y69A2A_2	y	N2	Small protein with similarity over N-terminal half to human MAD2 and S. cerevisiae Mad2p, a spindle-assembly checkpoint protein
ZC168.4	y	N2	Member of the cyclin protein family
ZK1127.1	y	N2	Protein that functions in development of the germ lineage, has similarity to D. melanogaster nanos
B0285.1	n		Serine/threonine protein kinase with strong similarity to cyclin-dependent protein kinases
C50F4.11	n		Coiled-coil protein with weak similarity to a family of D. melanogaster myosin heavy chains (see BLAST, see SMART), interacts with mdf-2 and is involved mitotic gen
C52D10.6	n		similarity to the SKP1 family of proteins, putative paralog of C. elegans C52D10.8 and C. elegans Y47D7A_138.D
F01G12.6	n		strong similarity to human Hs.179747 protein, a EVI5 homolog, a cell cycle regulator
F02E9.2A	n		Protein required for developmental timing of the ectoderm and cuticle; has weak similarity to H. sapiens YB1 gene product
F10C5.1	n		strong similarity to human CDC23 and S. cerevisiae Cdc23p, a component of the anaphase-promoting complex
T05A6.2	n		G1 phase cyclin-dependent kinase inhibitor; member of the CIP/KIP (p21/p27) family of cyclin-dependent kinase inhibitors
T12C9.4	n		moderate similarity to cyclins of human, S. cerevisiae, and D. melanogaster
T23F11.3	n		strong similarity over C-terminal half to human cyclin-dependent kinase 5 regulatory subunits 1 and 2 (p35/CDK5R1, p39/CDK5R2)
ZK1307.6	n		Putative ortholog of S. cerevisiae Cdh1p and of Drosophila fzr, proteins that are involved in cyclin desctruction
ZK520.4	n		Member of the cullin family of cell cycle control proteins
ZK856.1	n		Member of the cullin family of cell cycle control proteins
cdk-7			
F38H4.9			Member of the protein phosphatase protein family, predicted to be part of the PP2A core complex
H26D21.B			strong similarity to human HUS1 and S. pombe Hus1p cell cycle checkpoint protein
mdf-2			
plc2			
W01A6.E			Member of the cullin family of cell cycle control proteins
Y54G2A.A			

Daf-c Suppressor Screen

for

Cell Cycle Regulatory Mutants



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TGFβ-related pathways

roles in *Caenorhabditis elegans* development

Genetic and molecular analysis in *Caenorhabditis elegans* has produced new insights into how TGFβ-related pathways transduce signals and the developmental processes in which they function. These pathways are essential regulators of dauer formation, body-size determination, male copulatory structures and axonal guidance. Here, we review the insights that have come from standard molecular genetic experiments and discuss how the recently completed genome sequence has contributed to our understanding of these pathways.

In recent years, rapid progress has been made in understanding how transforming growth factor-β (TGFβ) and related ligands signal, in part because of a wealth of genetic and developmental information previously available on the pathways in which these ligands function. Model genetic systems show us how TGFβ-related pathways signal, how they are regulated and what cellular processes they control. As the *Caenorhabditis elegans* genome is completely sequenced and the tools to analyse these basic cellular processes is expanding, *C. elegans* will continue to play a major role in elucidating these functions and networks. In this review, we discuss the genetics and developmental biology of *C. elegans* TGFβ signaling.

The TGFβ superfamily plays critical roles in several important processes, such as cell proliferation, embryonic patterning and cell-type specification[1–6]. Biochemical identification of serine-threonine kinase receptors as mediators of TGFβ signaling was an important advance in the field, as was the identification of cytoplasmic and nuclear effectors belonging to the Smad family (a fusion of *sma* and *Mad* gene names). In *Drosophila*, Mothers against dpp (Mad) was genetically identified as part of the Decapentaplegic (dpp) pathway, and its cDNA sequence indicated it is a cytoplasmic protein, which is consistent with a role as a mediator of receptor signaling[7]. Work in *C. elegans* revealed three Smads that function in the same TGFβ signaling pathway, suggesting that multiple Smads might be required in other pathways[8]. Cloning of mammalian homologs demonstrated that these genes are conserved across diverse metazoan phyla[8,9]. Furthermore, developmental studies in *Xenopus* led to the identification of Smad2, a potent mesoderm inducer[10]. These discoveries spurred a flurry of Smad cloning and database harvesting.

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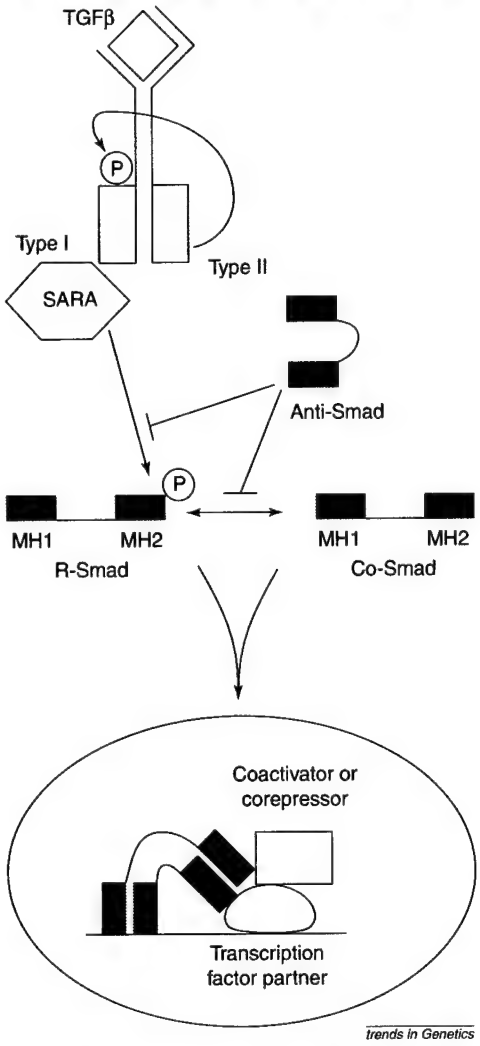
FIGURE 1. Model for TGFβ signal transduction



The Smads are depicted as two blocks connected by a linker region. The MH1 domains of the R-Smads and Co-Smads are shown in green and the MH1 domain of the Anti-Smad is shown in red. The MH2 domain of all the Smads is shown in blue. After ligand binding to the cellular receptors, Type I and Type II, the R-Smads are phosphorylated by the Type I receptor with the aid of Smad anchor for receptor activation (SARA). The phosphorylated R-Smad now complexes with the Co-Smad. Both enter the nucleus, where they bind specific DNA sequences. Because the Smads bind DNA weakly, their activity in the nucleus is altered by other transcription partners and other coactivators or corepressors. In most TGFβ pathways, an Anti-Smad is transcribed in response to signaling, which attenuates TGFβ signaling by binding to the receptors and/or Smads.

Smads come in three varieties

A general model for TGFβ superfamily signaling has been elucidated[1,3]. Ligand binding causes the type II receptor to phosphorylate the type I receptor (see Fig. 1). This event activates the type I receptor, which then, with the help of the SARA (for Smad anchor for receptor activation) protein[11], phosphorylates a Smad on C-terminal residues. Smads are defined by two conserved domains; MH1 (for Mad homology domain 1), which mediates DNA binding and some protein–protein interactions, and MH2, which mediates transcriptional activation and interactions with other transcription factors. R-Smads, which physically interact with distinct receptor complexes to generate signaling specificity, are activated when the type I receptor phosphorylates a conserved SSXS sequence at the C-terminus. These Smads interact with a Co-Smad, and the complex translocates to the nucleus where it, along with other factors, activates the transcription of target genes. The R-Smad or Co-Smad, or both, can interact with DNA via the MH1 domain. The Smad complex can activate or repress transcription, depending on whether it binds to a transcriptional activator or transcriptional repressor. In *Drosophila* and vertebrates, a single Co-Smad is used in all of the known pathways[1–4,12]. The Anti-Smads are a third class of Smads that are induced by TGFβ signaling and they block the phosphorylation of R-Smads and/or the interaction of R-Smads with Co-Smads.

Are Smads responsible for all signaling by the TGFβ family of receptors? The answer is 'perhaps'; the phenotypes of Smad mutants in *Drosophila* and *C. elegans* are virtually identical to the phenotypes of receptor mutants[7,8]. In addition, over-expression of Smads in *Xenopus* can mimic the effects of ligand and receptor, even in the presence of dominant-negative receptor constructs[4]. Thus, in these systems, Smads are necessary and sufficient for the major characterized effects of the receptors. However, this does not rule out the possibility of other molecules participating in signal transduction from the receptors, and it remains to be seen how substantially other molecules contribute to downstream events.

C. elegans dpp/BMP-related pathways

Now that the *C. elegans* genome has been sequenced, it provides a unique opportunity to examine the entire repertoire of TGFβ-related pathways in a way that has not been previously possible. Three TGFβ superfamily pathways have been genetically characterized in *C. elegans* (the dauer, Sma/Mab and *unc-129* pathways). The components of these pathways are most closely related to bone morphogenic protein (BMP) and dpp pathway components found in vertebrates and *Drosophila*. In addition, DAF-4, the only type II receptor in *C. elegans*, functions as a type II BMP2 receptor *in vitro*[13]. Therefore, we will refer to the *C. elegans* pathways as BMP-like pathways. This review discusses the elucidation of these three pathways, models for how signals in these pathways are transmitted, from production of the ligand to transcriptional changes in target cells, the role of these pathways in controlling development, and prospects for further understanding of this signal transduction pathway.

Dauer pathway

C. elegans, like many soil nematodes, can choose one of two larval developmental pathways. This developmental decision was the subject of a recent review[14], so we will focus on highlights that are relevant for an understanding of BMP-like signaling in this pathway. Chemosensory neurons measure food availability and competition for food resources from other nematodes[14]. Scarce food and high pheromone (a chemosensory cue that indicates population density) promote 'dauer' development. When this pathway is chosen, the worms arrest in the third larval

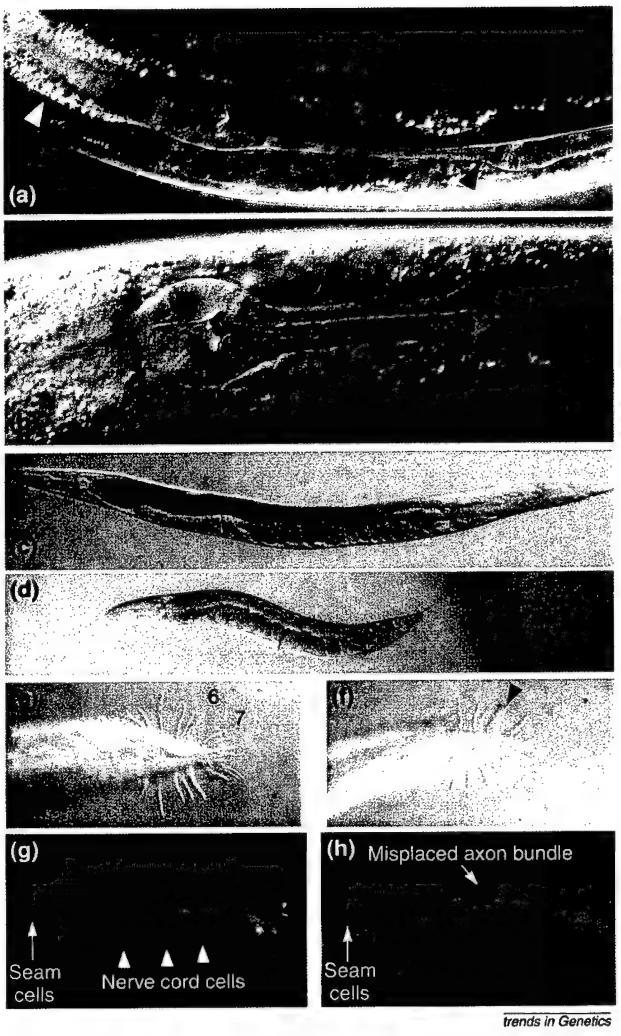
stage as a dauer, which has morphological, behavioral and physiological specializations that allow survival and dispersal from conditions in which food resources are inadequate to allow reproduction (Fig. 2a, b).

A BMP-like pathway plays a key role in the control of the dauer decision. Mutants that disrupt normal regulation of the dauer decision are of two types: dauer constitutive, which develop as dauers even under conditions that are appropriate for reproductive growth, and dauer defective, which develop as non-dauers under conditions that are appropriate for dauer development[14]. Careful analysis of phenotypes and epistasis relationships of the Daf mutants nicely predicted the functional relationships of the cloned genes[14,15]. In particular, a group of dauer-constitutive genes that constitute a BMP-like pathway was identified (Fig. 3). The genes encode a ligand (*daf-7*), two receptors (*daf-1* and *daf-4*), and two Smads (*daf-8* and *daf-14*)[2,14]. The biochemical relationships of these gene products have not been studied, but comparison with vertebrate gene products has led to a model that DAF-7 binds and activates the serine-threonine kinase receptor complex, which in turn phosphorylates the two Smads, DAF-8 and DAF-14. These Smads have an amino acid sequence that is related to the conserved SSXS motif that has been shown to be a target of the receptor kinases in other systems, but otherwise are highly diverged from other Smads (Table 1). DAF-8 and DAF-14 differ from R-Smads in the MH1 domain. DAF-8 is highly diverged in this domain[14], and is missing highly conserved residues that contact DNA and other residues that play important structural roles in the hydrophobic core of the MH1 structure[3]. DAF-14 is unique among Smads in that it has no MH1 domain[16].

The expression of the BMP-like ligand gene, *daf-7*, is controlled by cues received by chemosensory neurons. Chemosensory neurons in the amphid sensillum are critical for regulation of dauer[14]. Expression of a *daf-7*::green fluorescent protein (GFP) reporter is reproducibly seen only in the amphidal sensory neuron pair called ASI, and expression is activated by food and repressed by pheromone and high temperature[17–18]. Thus, coupling of environmental cues to regulation of the BMP-like pathway might be fairly direct; sensation of food and pheromone could take place in the sensory endings of ASI and be transduced to the nucleus to regulate *daf-7*. Alternatively, sensation could act in other amphid neurons and be transduced via interneurons or hormonal signals to ASI. Analysis of *ttx-3* mutants indicates that the temperature input for thermotaxis and dauer formation both use the same neuronal pathway[19], which does not include ASI; therefore, the effect of temperature on *daf-7* expression might be indirect. Mosaic analysis and expression of DAF-4 from cell-type specific promoters indicates that DAF-4 functions in the nervous system to control dauer formation[16]. DAF-7 ligand produced in ASI may bind DAF-4 receptor on nearby neurons to regulate the production of a hormonal signal that controls the dauer development of hypodermis, intestine, gonad and other cell types.

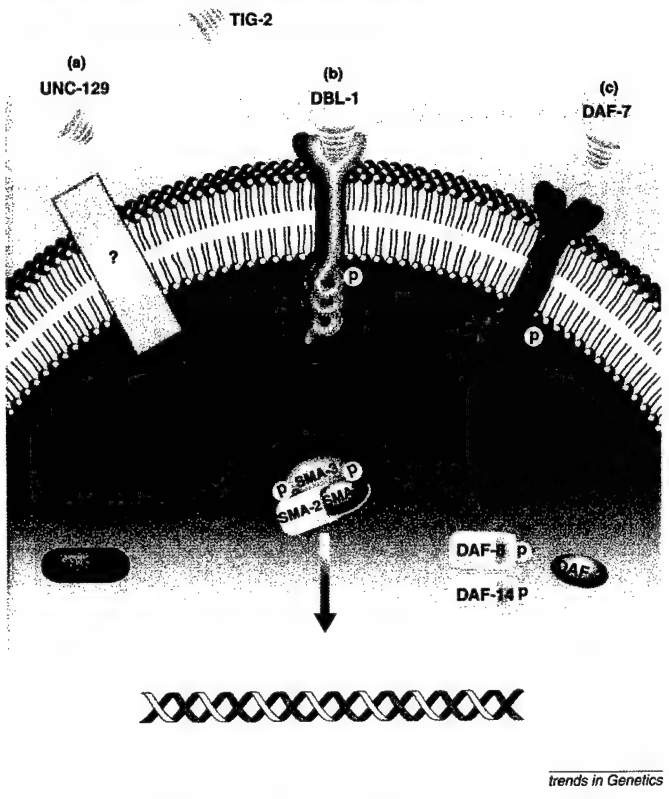
Two genes, *daf-3* and *daf-5*, were identified as dauer-defective mutations that suppress the dauer-constitutive phenotype of mutations in the five genes above[14]. This epistasis suggests that *daf-3* and *daf-5* act downstream or in parallel; in either event, the dauer-defective phenotype suggests that *daf-3* and *daf-5* are antagonized by the putatively receptor-activated Smads. *daf-5* has not been cloned, but *daf-3* encodes another Smad[20]. The fact that *daf-3* is antagonized by the BMP-like pathway genes is unique. Anti-Smads have been described, but their function is to antagonize the receptors and receptor-activated Smads, and they have no known function in the absence of the receptors or Smads. By contrast, *daf-3* is antagonized by the receptors and Smads, and functions to induce dauer formation when the receptors or Smads are missing in mutants. DAF-3 is like other Smads in structure (Table 1),

FIGURE 2. Phenotypes exhibited by animals mutant for the BMP-like pathways



(a) Anterior of *daf-7* mutant induced to form dauer, (b) *daf-7, daf-3* mutant non-dauer L3. Note that the width of the body is much less in the dauer than in the L3 animal, as is the pharynx (solid arrowheads show the outline of the pharynx). The dauer also has a sparkly intestine (open arrowhead), probably owing to the accumulation of storage granules. (c) A wild-type worm. (d) A *sma-6* mutant at the same magnification. (e) A wild-type male tail. Rays 6 and 7 are marked. (f) A *sma-6* tail. The arrowhead shows the fat ray produced by the fusion of rays 6 and 7. (g) and (h) represent the midbody region of a wild type and *unc-129* mutant, respectively. Ventral cord cells are expressing green fluorescent protein. Note that the *unc-129* animal has an ectopic nerve bundle due to misdirected axon migrations. Panels c–f are reproduced with the permission of Company of Biologists Ltd[29]. Panels g–h are reproduced with permission from Academic Press[42].

FIGURE 3. *C. elegans* BMP-like pathways



The pathways used in (a) axon pathfinding, (b) body size/male tail development, and (c) dauer formation. Ligands are shown outside the cell in pink. Receptors are shown in green (receptors for UNC-129 are unknown).

despite its functional distinctiveness – it has both MH1 and MH2 domains[20] and it can bind DNA[21].

A model to explain these unique features has been proposed[20], in which phosphorylation of DAF-8 and DAF-14 by the receptors leads to their physical interaction with DAF-3. To explain the epistasis relationship, this interaction is suggested to inactivate DAF-3. When the DAF-8 and DAF-14 Smads are not phosphorylated, or are not present in mutants, DAF-3 would be free to regulate gene expression. It is also possible that the DAF-8 and DAF-14 Smads signal in parallel to DAF-3 without direct interaction, and that the two pathways are mutually antagonistic[14].

daf-12 mutates to a dauer-defective phenotype, and is epistatic to mutations of the *daf-7* BMP-like pathway. *daf-12* encodes a nuclear hormone receptor homolog, and is most closely related to the vertebrate Vitamin D receptor and a *Drosophila* orphan receptor, DHR96, which is ecdysone induced[22]. Smad3 and Vitamin D receptor have recently been shown to physically interact and to coactivate Vitamin D-responsive promoters[23]. So DAF-12 could well be a cofactor of the DAF3 Smad. However, *daf-12* mutations have heterochronic effects (those affecting developmental timing) that are not shared by *daf-3* and *daf-5*, in that *daf-12* mutants repeat second larval stage patterns of cell division and migration during the third larval stage[24]. In addition, *daf-12* is epistatic to *daf-28*, whereas *daf-3* and *daf-5* are not[25]. These facts, and the homology of DAF-12 to a *Drosophila* protein that binds ecdysone and that might be involved in molting[22], suggest that *daf-12* might not be an integral part of the BMP-like pathway. Rather, *daf-12* might be involved in progression through the L2 stage to L3, and *daf-12* mutants might have a defect in carrying out third larval-stage fates that includes an inability to form the specialized L3 dauer. A precedent can be found for heterochronic mutations affecting dauer formation in a manner that is apparently independent of BMP-like signals. The gene *lin-4* is similar to *daf-12* in that it is heterochronic, unable to form dauers and epistatic to *daf-7* (Ref. 26). The function of *lin-4* is to negatively regulate *lin-14*, a novel gene that has no known connection to BMP-like signaling.

Sma/Mab pathway

As described above, studies on the dauer pathway established the existence of BMP-like signaling in *C. elegans*. Interestingly, *daf-4*, which encodes a type II BMP2/BMP4 receptor[13], has mutant phenotypes not exhibited by the other *daf* genes. In addition to producing dauer constitutive animals, mutations in *daf-4* also cause a small body size (Sma; see Fig. 2c, d), crumpled spicules, and male sensory tail ray defects (Mab; see Fig. 2e, f)[8]. The spicules are male copulatory organs that aid sperm transfer, and the male sensory rays are used for locating the vulva. A model to explain the multiple mutant phenotypes of *daf-4* is that DAF-4 propagates two distinct BMP-like signals in *C. elegans* to control the dauer pathway and the Sma/Mab pathway (Fig. 3). This model requires that two sets of ligands and type I receptors act with DAF-4 to transduce signals via distinct Smads, resulting in different developmental responses. Cloning of genes with a Sma/Mab phenotype demonstrated this to be true[8,27–29].

Three genes (*sma-2*, *sma-3* and *sma-4*) that mutate to cause a small body size, crumpled spicules, and male tail ray defects were found to encode proteins similar to each other[8]. Moreover, they were homologs of the *Mothers against dpp* (*Mad*) gene from *Drosophila*[7], the first identified member of this family. If these Smads are regulated by DAF-4, then there should be another type I receptor which forms a complex with DAF-4. A polymerase chain reaction screen using primers similar to sequences of *Drosophila* BMP-like type I receptors[29] identified a new type I receptor that was a candidate partner for DAF-4. This receptor corresponds to *sma-6*, which mutates to a Sma/Mab phenotype similar to that of *daf-4*, *sma-2*, *sma-3* and *sma-4*. These results confirm that *daf-4* sends two independent signals and provides *in vivo* evidence that a given type II receptor can interact with different type I receptors (Fig. 3). A mutant screen for additional small animals and a reverse genetic approach showed that null mutants in the BMP-like ligand gene *dbl-1* (also known as *cet-1*) exhibit the same mutant phenotypes as the Smads and the type I receptor, *sma-6* (Refs 27, 28). A *dbl-1*::GFP fusion is mainly expressed in the amphid neurons, neurons of the ventral nerve cord and neurons and glial cells in the tail, which must serve as the source of the signal for some or all of the three developmental processes in which it is involved.

This BMP-like pathway appears to regulate body size by a novel mechanism. Newly hatched *Sma* mutant animals are normal length, but *Sma* mutant adults are about

TABLE 1. Comparisons of dauer pathway Smads with other known Smads

	MH1 domain	MH2 domain	SSXS phosphorylation target	Relationship to receptors	Refs
R-Smads (includes Smad1, 2, 3, 5, 8 from vertebrates; Mad from *Drospholia* and SMA-2 and SMA-3 from *C. elegans*)	Yes	Yes	Yes	Activated by receptors	1–6
Co-Smads (includes Smad4 from vertebrates, MEDEA from *Drosophila*, and SMA-4 from *C. elegans*)	Yes	Yes	No	Assists R-Smads in transducing signal	1–6
Anti-Smads (includes Smad6, 7 from vertebrates and Dad from *Drosophila*)	[a]Diverged	Yes	No	Antagonizes receptors	1–6
DAF-8	Diverged	Yes	Yes	Activated by receptors?	14
DAF-14	No	Yes	Yes	Activated by receptors?	16
DAF-3	Yes	Yes	No	Antagonized by receptors	19

[a]The diverged MH1 domain in Anti-Smads and DAF-8 is not likely to bind DNA, because it is missing structural residues that form the hydrophobic core and contact DNA.

half the size of normal adults[27,28]. Examination of adult nuclei has not shown any differences in number from wild type, strongly suggesting that the small body size is not caused by a decrease in cell number[27,28]. This would indicate that all the expected cells are present but some or all are physically smaller than in the wild type. An interesting possibility is that the controls that regulate cell growth and mitosis have been uncoupled in the *Sma* mutants so that the cells divide before their normal dividing volume is reached. The receptors and Smads might control the production of a signal that regulates cell division, or might function cell autonomously in various cells to control the size of those cells. The broad expression reported for *daf-4* and *sma-6* are consistent with either model[20,29].

Null mutations in the Sma/Mab pathway result in transformations of rays and often fusion between adjacent rays. The rays that are transformed in Sma/Mab mutants are mostly dorsal rays 5, 7 and 9, which adopt the fate of their anterior neighbor and often fuse with it, creating a fatter ray[27–29]. The basis for these phenotypes appears to be an improper migration of the cells that comprise the ray. Mutations in the Sma/Mab genes also disrupt a cellular migration necessary to form the spicule, a copulatory structure necessary for mating[30].

Do *C. elegans* BMP-like pathways pattern the embryo?

BMP and dpp pathways are essential for vertebrate and *Drosophila* development; mutants die as embryos. It has been suggested that *Drosophila* and vertebrates have homologous BMP pathways that control dorsoventral patterning in the embryo[31]. The defects in axon outgrowth in *unc-129* mutants and in male tail rays in Sma/Mab mutants can be interpreted as defects in dorsoventral patterning. However, no BMP-like pathway mutants disrupt the basic dorsoventral axis of *C. elegans* in the manner of *Drosophila* or vertebrates. Is it possible that the *C. elegans* BMP pathways function in dorsoventral patterning in early embryogenesis? We think this is unlikely, for two reasons. First, one of the *daf-4* mutant alleles has a stop codon that is predicted to eliminate the last 45 amino acid residues of the kinase domain[13]. This allele of *daf-4* probably encodes a kinase-inactive protein, yet it has no defects other than the Daf and Sma/Mab phenotypes we have discussed. There is no other type II receptor in the *C. elegans* genome sequence that could function in the place of DAF-4. In addition, analysis of *sma-6* and *sma-3* mutants also indicates that molecular null alleles of these genes have been isolated, and no embryonic phenotypes have been observed. Analysis of double mutants of BMP-like pathway genes provides the second reason for our suggestion that there is no embryonic function for these pathways. Our reasoning is as follows. It is possible that the embryonic function of the genes is not apparent in the single mutants because the screens that led to their isolation were for larval phenotypes. These screens might have led to the isolation of hypomorphs only. However, if this were the case, we expect that the embryonic function might be revealed when two of these putative hypomorphs are combined. Many double mutants have been made between alleles of multiple genes in the dauer pathway[15] and Sma pathway[29], as well as double mutants that include mutations in both pathways[27,29], and none of these double-mutant combinations show any enhancement of known phenotypes or any new embryonic phenotype.

What does the lack of embryonic function for the BMP-like pathways in *C. elegans* imply about the evolution of these pathways? It is possible that the common ancestor of *C. elegans*, *Drosophila* and vertebrates had a BMP pathway functioning in dorsoventral patterning, and the pathway was lost in an ancestor of *C. elegans*. It is also possible that *Drosophila* and vertebrates share a common ancestor more recently than with *C. elegans*, and that the BMP pathway function arose in the *Drosophila*/vertebrate line after its divergence from the *C. elegans* line. It is not possible to be certain which model is true; the relationship of nematodes to other phyla is uncertain, because of a lack of fossil evidence and the rapid rate of sequence evolution in many nematode species[32]. A recent analysis of 18S RNA sequences suggests that arthropods and nematodes are more closely related to each other than either is to vertebrates[33], which would imply that the first model above is correct.

Orphan genes

Mutations in *unc-129* cause defects in axonal guidance, and *unc-129* encodes a BMP-like ligand[34]. *unc-129* mutants do not have Daf or Sma/Mab phenotypes, and examination of *daf-1*, *sma-6* and *daf-4* mutants revealed no axon guidance defects. The genome sequence reveals no additional receptors or Smads that could constitute an *unc-129* signal transduction pathway. Thus, *unc-129* is not likely to signal in the conventional way. It has been suggested that UNC-129 might function by binding

UNC-5 directly or by acting on a signal transduction pathway unlike the conventional BMP-like pathways. The genome sequencing consortium has identified one other Smad in the *C. elegans* genome with an unknown function; it is most closely related to an Anti-Smad, but its biochemical properties have not been examined. The genome sequence also reveals an orphan ligand of unknown function, *tig-2*. All of the receptors and other Smads in the genome participate in Sma/Mab or Daf pathways, so it seems more likely that the new Anti-Smad and orphan ligand function in either or both of the characterized pathways, rather than in a new, undiscovered pathway. The genome sequence reveals a SARA homolog. The characterized vertebrate SARA proteins function with the activin/TGFβ pathway specific Smad2 and Smad3 (Ref. 11). The existence of SARA in *C. elegans*, which has only BMP-like pathways, suggests the possibility that these pathways in vertebrates use a different isoform of SARA that remains to be discovered and characterized.

Smad cofactors

The forkhead protein FAST1 forms a DNA-binding complex with Smads in vertebrates[1–4], and there are three *C. elegans forkhead* homologs that regulate the same processes as the Sma/Mab and dauer BMP-like pathways. The *daf-16* gene mutates to a dauer-defective phenotype that is epistatic to the dauer-constitutive phenotype of mutations of the DAF-2 insulin receptor and associated signaling molecules. DAF-16 is a forkhead transcription factor[35,36], and therefore might interact directly with DAF-3. It should be noted that DAF-16 is not orthologous to FAST1, but to AFX and FKHRL-1. These two proteins have recently been shown to be regulated by the mammalian homolog of AKT-1 and AKT-2 (Refs 37, 38), which were genetically identified as regulators of *daf-16* (Ref. 39). A second forkhead protein, LIN-31, has been implicated in the formation of the spicule. *lin-31* and Sma/Mab pathway mutants have identical phenotypes in spicule development; the migration of a tail cell and the resulting production of a cellular mold for the spicules, is abnormal[30]. Thus, LIN-31 might be a cofactor of SMA-2, SMA-3 and SMA-4 in controlling this migration. *lin-31* mutants do not affect the ray or body-size phenotypes seen in *sma-2*, *sma-3* and *sma-4* mutants, so any interaction is tissue specific.

The Smad DAF-3 and the forkhead protein PHA-4 regulate expression of a pharyngeal promoter, but these two proteins might not be cofactors. PHA-4 is required during embryogenesis for organogenesis of the pharynx, and induces expression of the myosin gene, *myo-2* (Refs 40, 41). By contrast, DAF-3 plays no detectable role in embryonic development of the pharynx, but does bind an element from the *myo-2* promoter and represses expression in adults[21]. DAF-3 could exert its repressive effect by binding and inactivating PHA-4.

Conclusions and future prospects

Many of the major players in these pathways have recently been identified. Probably the most important questions to answer next are: where and when do these pathways act, and how do these pathways interact with other pathways? In particular, which cells receive the signals, and how do they respond? Additional mutations that disrupt each of the three pathways have been isolated, and cloning these genes will help to answer the outstanding questions. The identification of Smads in the dauer and Sma/Mab pathways suggests that a major output is transcriptional, and it will be important to identify the Smad target genes. An additional promising approach to identifying genes transcriptionally regulated by the Smads is to use a microarray that contains sequences corresponding to all of the *C. elegans* coding regions, similar to the sort of array that has been used to study the expression of genes in yeast. The existence of the genome sequence also makes it possible to screen for target genes '*in silico*', by identifying genes that have Smad-binding sites upstream. Given that every gene in the TGFβ pathway is conserved between phyla, new insights are likely to come from studying the developmental processes involving TGFβ-like pathways in *C. elegans*. The coming years should continue to provide new insights into how these signaling pathways function, and perhaps they will provide a fairly complete picture of all TGFβ superfamily signaling in a single intact organism.

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Drosophila in cancer research

an expanding role

In recent years, *Drosophila* researchers have developed powerful genetic techniques that allow for the rapid identification and characterization of genes involved in tumor formation and development. The high level of gene and pathway conservation, the similarity of cellular processes and the emerging evidence of functional conservation of tumor suppressors between *Drosophila* and mammals, argue that studies of tumorigenesis in flies can directly contribute to the understanding of human cancer. In this review, we explore the historical and current roles of *Drosophila* in cancer research, as well as speculate on the future of *Drosophila* as a model to investigate cancer-related processes that are currently not well understood.

In 1916, decades before *Drosophila* would become one of the most popular models for studying many aspects of modern biology, the discovery of melanotic tumor-like granules in mutant larvae by Bridges and Stark first suggested that flies could develop tumors[1]. Later, spontaneous mutations were identified that caused animals to die at larval stages with overproliferation of certain internal tissues[2,3]. Subsequent screens for such a phenotype were highly successful as dozens of genetic loci were recovered in *Drosophila* at a time when few human tumor suppressors had been identified[2,4–6]. Most of the tumor-causing mutations that were identified during this time were defined as tumor suppressor genes because they behaved as recessive loss-of-function mutations[7]. Molecular characterization of some of these fly tumor suppressor genes pointed to the importance of cell–cell communication in the regulation of cell proliferation[3,8,9] (Table 1).

Despite very promising beginnings, the fly has not received much attention as a model system for cancer research. Several factors might have contributed to this outcome. Although the over-proliferated larval tissues and melanotic tissues that were observed in the fly mutants had some characters resembling those of human tumors, they lacked the appearance of the massive *in situ* overproliferation that is commonly associated with most mammalian tumors. Second, the molecular characterization of these early fly tumor suppressors did not demonstrate a similarity to the tumor suppressors that had been identified in humans[10,11]. Furthermore, characterization of these fly tumor suppressor genes did not provide an obvious connection to the contemporary understanding of the processes that are involved in tumor formation, such as regulation of the cell cycle. Finally, the indiscreet classification of some *Drosophila* genes as tumor suppressors also contributed to the state of neglect by the general cancer research community. For example, inactivation of neurogenic genes causes hypertrophy of the nervous system. However, they are not tumor suppressors because the phenotype is caused by conversion of epidermal cells into neurons and not by overproliferation of neuronal tissues[12].

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Review

Transforming growth factor β signaling mediators and modulators

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Abstract

Transforming growth factor β is a multi-functional growth and differentiation factor responsible for regulating many diverse biological processes in both vertebrate and invertebrate species. Among the most dramatic of TGFβ's effects are those associated with specification of cell fates during development and inhibition of cell cycle progression. The core TGFβ signaling pathway has now been described using a synergistic combination of genetic and biochemical approaches. Transmembrane receptors with intrinsic protein serine kinase activity bind ligand in the extracellular milieu and then phosphorylate intracellular proteins known as Smads. Phosphorylated Smads form heterooligomers and translocate into the nucleus where they can modulate transcriptional responses. More recent studies indicate that many other proteins serve as modulators of Smad activity, and utimately define specific cellular responses to TGFβ. Here we describe both the simplistic core TGFβ signaling pathway and the growing number of proteins that impinge on this pathway at the level of Smad function to either enhance or inhibit TGFβ responses

Keywords: Receptor serine kinases; Signal transduction; Smads; Transcription factors; Tumor suppressors

1. Introduction

The transforming growth factors β (TGFβs) are extracellular peptides that regulate many diverse biological processes. TGFβs were initially identified and named based on the observation that they stimulate cellular proliferation of fibroblasts in culture. However, it was shown later that these molecules are among the most potent known inhibitors of cellular growth and division in many other cell types. In fact, TGFβs are now the most well-studied and widely known family of growth inhibitory proteins; molecular components at each step in the signaling pathway have been identified as tumor suppressors. TGFβs also have roles in regulating cellular differentiation, adhesion, motility, and death (Massagué, 1990; Roberts and Sporn, 1990, 1993; Alexandrow and Moses, 1995).

Many other peptide growth factors are structurally related to TGFβ and are, therefore, members of the TGFβ-superfamily of ligands. These include the activins, the bone morphogenetic proteins (BMPs), and the growth and differentiation factors (GDFs) among others. In addition, homologues have been identified in *Drosophila melanogaster* (*dpp*, *Gbb/60A*, *screw*, and *dActivin*), and in *Caenorhabditis elegans* (*daf-7*, *dbl-1*, *unc-129*, and *tig-2*). These factors have essential roles in embryonic patterning, organogenesis, immune system function, and tissue homeostasis (Kingsley, 1994; Raftery and Sutherland, 1999; Patterson and Padgett, 2000).

Remarkable progress has been made over the last several years in elucidating the molecular mechanisms by which cells translate the presence of TGFβ in the

Abbreviations: ALK, activin receptor-like kinase; BMP, bone morphogenetic protein; CBP, CREB-binding protein; dpp, decapentaplegic; EGF, epidermal growth factor; FAST, Forkhead activin signal transducer; Gbb, glass bottom-boat; GDF, growth and differentiation factor; HDAC, histone deacetylase; HGF, hepatocyte growth factor; Mad, mothers against dpp; MH1, Mad homology domain 1; MH2, Mad homology domain 2; PAI-1, plasminogen activator inhibitor-1; PCR, polymerase chain reaction; RSK, receptor serine kinase; SARA, Smad anchor for receptor activation; TGF, transforming growth factor.

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extracellular milieu into discrete changes in gene expression. A combination of biochemical, cell biological, and genetic analyses were not only exploited, but were absolutely necessary, in developing the current models for TGFβ signal transduction. These studies have defined a novel signaling strategy in which ligand-regulated transmembrane receptor serine kinases (RSKs) modulate the activity of transcriptional regulators called Smads. This work, and the resulting models, has recently been well-reviewed both comprehensively (Massagué, 1998), and with specific focus on the role of these factors in the process of development (Whitman, 1998), and in genetic model organisms (Raftery and Sutherland, 1999; Patterson and Padgett, 2000). Therefore, this review will describe the core TGFβ signaling pathway in brief historical context, and then focus on recent work that demonstrates the importance of co-factors and cross-talk among different signaling pathways in modulating TGFβ signal transduction.

2. TGFβ signal transduction

The first milestone in the molecular analysis of TGFβ signaling pathways was the identification and cloning of a type II receptor for activin, and soon after for TGFβ (Mathews and Vale, 1991; Lin et al., 1992). These discoveries were of special importance because each of the receptors contained an intracellular protein kinase domain with predicted serine/threonine specificity. This observation suggested the existence of a novel intracellular signaling strategy because all previously characterized receptor protein kinases were tyrosine specific. Subsequent cloning of type II receptors for other members of this ligand superfamily, including homologues in *Drosophila* and *C. elegans*, proved that these receptors are also members of a highly conserved protein family, the type II RSKs (Georgi et al., 1990; Childs et al., 1993; Baarends et al., 1994; Kawabata et al., 1995).

2.1. RSKs

Analysis of a panel of mutagenized mink lung epithelial cell lines strongly suggested that another cell surface binding protein for TGFβ played an essential role in signal transduction (Wrana et al., 1992, 1994). These proteins were cloned using PCR strategies based on the conserved sequences within the kinase domains of the type II receptors. The most comprehensive description of this group of related molecules designated them activin receptor-like kinases (ALKs); therefore, this nomenclature has been used until functional names could be assigned (Franzén et al., 1993; ten Dijke et al., 1993, 1994). Although these proteins share homology with type II RSKs, they clearly belong to a distinct family and have unique structural features, which now define type I RSKs. Most notable is the presence of a conserved glycine- and serine-rich sequence motif (GS domain) between the transmembrane and kinase domains that contributes to the activation state of the receptors.

Demonstration that type II and type I RSKs form ligand-induced heteromeric complexes at the cell surface was the first step in defining the mechanism of RSK activation and signal initiation (Wrana et al., 1992). Subsequently, unidirectional phosphorylation of the type I RSKs at the GS domain by type II RSKs was shown to be required for signal propagation (Wrana et al., 1994). The fortuitous identification of an activating point mutation (T204D in ALK5, T206E in ALK4) in type I receptors proved very useful in confirming the order of events in RSK activation (Wieser et al., 1995; Willis et al., 1996). For example, these activated type I receptors induce ligand-independent signaling in the absence of functional type II RSKs, and in the presence of dominant-negative type II RSK mutants (Wieser et al., 1995; Attisano et al., 1996; Willis et al., 1996). Therefore, type I receptors are downstream of type II receptors in RSK-mediated signaling. (This stands in sharp contrast to the mechanism of activation of receptor tyrosine kinases, in which ligand induces homomeric receptor complexes, and bidirectional transphosphorylation of receptor subunits initiates signaling.)

Therefore, the following model for initiation of RSK-mediated signaling has been proposed. A TGFβ-family ligand induces heteromeric RSK complex formation at the cell surface. Type II RSKs phosphorylate type I RSKs at serines within the GS domain, which results in activation of the type I kinase and subsequent phosphorylation of intracellular signal mediators.

Because there appear to be many more ligands within the TGFβ-superfamily than identified RSKs, and because some overlap has been observed in ligand binding by RSKs, the question of ligand-receptor specificity is still being investigated. Initially, type II receptors were thought to determine the ligand binding specificity of the RSK complex; TβRII, for example, binds only to TGFβ (Lin et al., 1992). However, other type II RSKs bind to multiple ligands in combination with different type I receptors. ActRII binds to activin in combination with ALK4, but also binds both BMP2 and BMP7 in the presence of BMP type I receptors (Cárcamo et al., 1994; Yamashita et al., 1995; Willis et al., 1996). In addition, ActRIIs have been shown to have functional roles in mediating both activin and BMP signals in *Xenopus* (Chang et al., 1997).

Recently, the combination of genetic analysis and completion of the genome sequence of *C. elegans* has provided significant insight regarding this issue. The key observation was that worms mutant for type I RSK *daf-1* displayed only a subset of the mutant phenotypes observed when the type II RSK *daf-4* was mutated (Georgi et al., 1990; Estevez et al., 1993). This suggested

that *daf-4*-mediated signals in addition to those propagated via *daf-1*. Would these additional signals be independent of type I receptors, or could *daf-4* pair with other type I receptors in addition to *daf-1*? The answer came with the cloning and analysis of *sma-6*, a type I RSK in worms that accounted for all of the *daf-4* phenotypes not associated with *daf-1* (Krishna et al., 1999). This proved that a single type II RSK could function in parallel pathways with different type I RSKs in worms. Furthermore, genome sequencing indicates with near certainty that these are the only available RSKs in *C. elegans*. Given the high degree of conservation among TGFβ pathway components between nematodes and vertebrates, it is quite likely that type II RSKs function similarly in mammalian systems.

2.2. Smad proteins

While the mechanism of signal initiation and ligand-receptor specificity was being investigated, an intensive search was also being carried out to identify and clone molecular mediators of RSK signaling. Because the predicted substrate specificity of the RSKs differs from that of the well-characterized receptor tyrosine kinases, the mediators of RSK signaling were expected to be novel. Genetic analysis in both *Drosophila* and *C. elegans* yielded the first bona fide members of this signaling pathway, which are now known as Smads (Fig. 1).

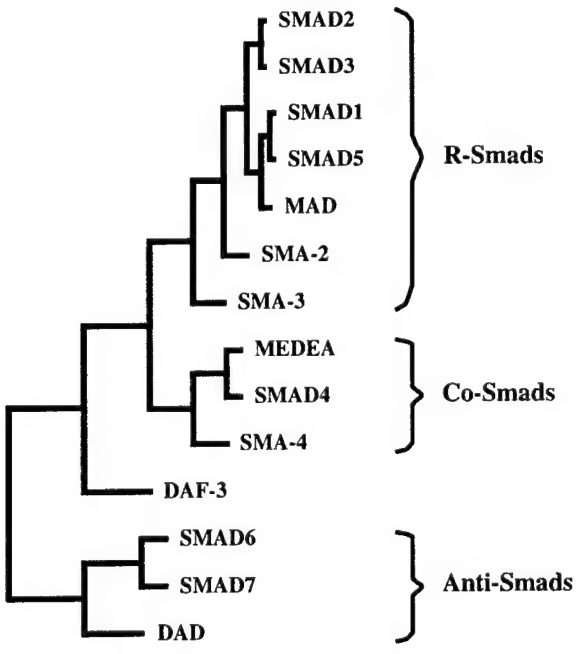

Fig. 1. Phylogenetic relationships among Smad proteins. Smad proteins fall into three classes based on sequence homology, which correlate with their observed functional characteristics. The only exception is DAF-3; its sequence more closely resembles that of Co-Smads, and its function is divergent.

The observation that different mutations in the *Drosophila* BMP homologue, *dpp*, result in phenotypes of graded severity was exploited in screens designed to isolate modifiers of weak *dpp* alleles. Two maternal effect enhancers of a weak *dpp* phenotype were isolated and named *Mothers against dpp* (*Mad*) and *Medea* (Raftery et al., 1995; Sekelsky et al., 1995). Molecular cloning and sequencing of *Mad* indicated that the gene encoded a protein with no previously described functional motifs, although the sequence was homologous to three predicted open reading frames in the *C. elegans* genome. Subsequent cloning of the worm genes (*sma-2*, *-3*, and *-4*) based on shared mutant phenotypes of small body size and male tail defects with type II RSK *daf-4* confirmed the presence of these homologous proteins, and demonstrated that multiple Smads are required in this signaling pathway (Savage et al., 1996). Although this protein family does not share homology with other known proteins, each member does contain highly conserved N- and C-terminal domains separated by a linker of variable length and sequence. The N-terminal domain has been called Mad-homology domain 1 (MH1), and the C-terminal domain has been designated Mad-homology domain 2 (MH2) (Fig. 2). Because these proteins lacked identifiable hydrophobic signal sequences or transmembrane domains, and because genetic mosaic analysis indicated that *sma-2* is required cell autonomously in the same cells as *daf-4*, they were proposed to function as intracellular mediators of RSK signaling (Savage et al., 1996).

Following the genetic identification of these molecules, several vertebrate homologues were rapidly cloned (Fig. 1). One of the first human *Mad* homologues, *DPC4*/Smad4, was cloned independently in a search for genes homozygously deleted in pancreatic carcinoma; this observation led to the suggestion that these genes may function as tumor suppressors (Hahn et al., 1996). In addition, a murine homologue was cloned based on its functional capacity to change cell fate in *Xenopus* from ectoderm into mesoderm (Baker and Harland, 1996). A unified nomenclature was soon adopted in which the original gene names from *C. elegans* (*Sma*) and *Drosophila* (*Mad*) were combined; the proteins are now known as Smads (Derynck et al., 1996).

Functional analysis of Smads in *Drosophila* and in *Xenopus* further elucidated their role in mediating RSK signals downstream of receptors. In addition to the observations made in worms, genetic evidence for placing Smads downstream of receptors was provided by the demonstration that *Mad* mutants can suppress the effects of dominant activating mutations in the type I RSK *thickveins* (Hoodless et al., 1996; Wiersdorff et al., 1996). Analysis of Smad function in *Xenopus* animal cap explants demonstrated that overexpression of Smad1 resulted in ventralization reminiscent of treatment with BMPs; conversely, overexpression of Smad2 resulted in

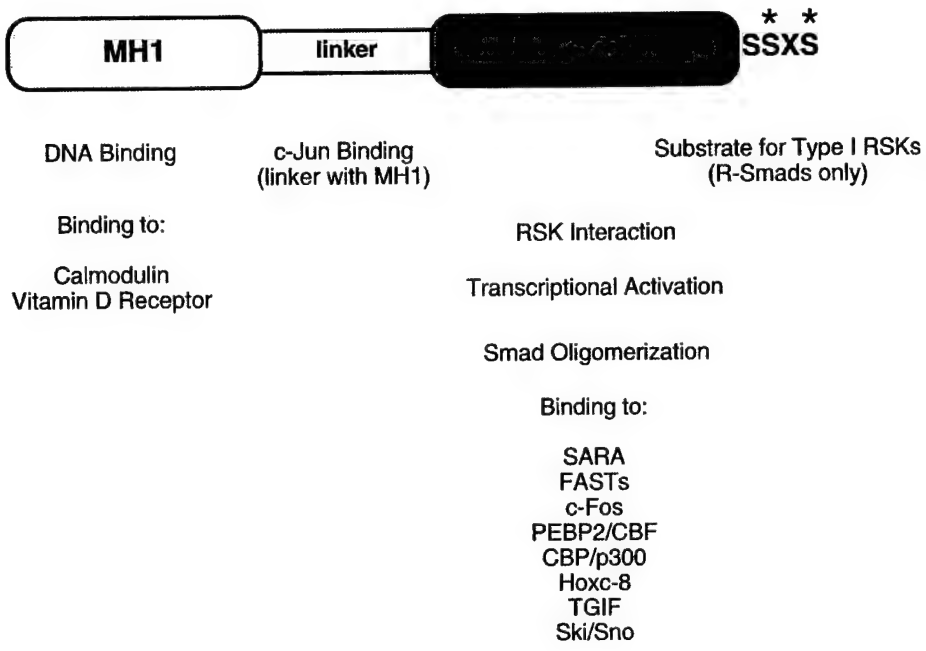

Fig. 2. Structural and functional characteristics of the Smad proteins. Highly conserved *Mad* homology domains 1 and 2 are shown in schematic form. Stars mark the type I RSK phosphorylation sites within the R-Smad C-terminal sequence. Functional roles of each domain are listed below the schematic representation.

activin-like mesodermal dorsalization (Baker and Harland, 1996; Graff et al., 1996). Observation of similar effects for Smad 1 and Smad2 in the presence of dominant-negative receptors provides additional evidence that Smads act downstream of receptors (Graff et al., 1996). These results not only demonstrated that Smads mediate TGFβ-family signals, but also provided the first evidence that different Smads are responsible for transducing different ligand-specific responses (Table 1).

Rapid progress in the biochemical analysis of Smad proteins followed their identification and biological characterization. The first question to be addressed, based on the predicted enzymatic activity of the receptors, was whether Smads become phosphorylated in response to receptor activation. In fact, one class of Smads is phosphorylated directly by type I RSKs on extreme C-terminal serines within the terminal sequence motif SSXS (Hoodless et al., 1996; Macias-Silva et al., 1996; Souchelnytskyi et al., 1996; Kretzschmar et al., 1997b). This group of Smads is known as the 'receptor-regulated' class (R-Smads) and, when phosphorylated, physically associates with another class of Smads known as the 'common' Smads (Co-Smads) (Lagna et al., 1996). Co-Smads lack the SSXS motif and are not phosphorylated by RSKs in response to ligand (Zhang et al., 1996). Therefore, ligand-induced phosphorylation of R-Smads results in heteromeric complex formation with Co-Smads in the cytoplasm.

Specificity between TGFβ-family signaling pathways is also maintained at the level of R-Smad phosphorylation (Table 1). Smads 1, 5, and 8 are phosphorylated only by BMP type I receptors (Hoodless et al., 1996),

Table 1
Molecular mediators of TGFβ-family signaling pathways. Known components of selected ligand-initiated cascades are listed. This is not a comprehensive list, but rather an ordering of the most well-characterized pathways

Ligand	Type II RSK	Type I RSK	R-Smad	Co-Smad
TGFβ	TβRII	ALK5	Smad2 Smad3	Smad4
Activin	ActRII ActRIIB	ALK4	Smad2 Smad3	Smad4
BMP 2/4	BMPRII ActRII	ALK3 ALK6	Smad1 Smad5 Smad8	Smad4
dpp	*punt*	*thickveins* *saxophone*	*Mad*	*Medea*
dActivin	*punt*	*baboon*	*dSmad2*	*Medea*
daf-7	*daf-4*	*daf-1*	*daf-8* *daf-14*	*daf-3* (?)[a]
dbl-1	*daf-4*	*sma-6*	*sma-2* *sma-3*	*sma-4*

[a] *Daf-3* is structurally similar to the Co-Smads, but functions differently as described in the text.

whereas Smads 2 and 3 are phosphorylated specifically by TGFβ and activin type I receptors (Eppert et al., 1996; Macias-Silva et al., 1996; Zhang et al., 1996). This specificity is the result of conserved recognition sequences within both the type I RSKs and the R-Smads (Feng and Derynck, 1997; Chen et al., 1998; Lo et al., 1998).

The next clue as to the possible function of these unique signal mediators was the observation that RSK signaling resulted in nuclear accumulation of phosphorylated R-Smad and Co-Smad heteromers (Macias-Silva et al., 1996; Liu et al., 1997; Nakao et al., 1997b). Analysis using Smad4-deficient cell-lines indicated that association with Smad4 is not required for nuclear translocation of R-Smads (Liu et al., 1997). However, Co-Smads do not accumulate in the nucleus without a phosphorylated partner, demonstrating that Co-Smads require an activated R-Smad for nuclear entry (Liu et al., 1997; Das et al., 1998). Therefore, if RSK activation and R-Smad phosphorylation results in nuclear accumulation of heteromeric Smad complexes, then it was reasonable to expect that Smads would have some function in the nucleus.

Once again, a combination of experimental systems was exploited to demonstrate a functional role for Smads inside the nucleus. First, the MH2 domains of both Smad1 and Smad4 were shown to activate transcription in cultured cells when tethered to DNA as GAL4-DNA binding domain fusions (Liu et al., 1996). In addition, full-length Smad1 exhibited BMP-induced transcriptional activity in the same assay. These observations not only supported the idea that Smad proteins could be direct transcriptional regulators, but also suggested that Smads may be the only required mediators of TGFβ-family signals between RSKs and the nucleus.

The hypothesis that Smads might be physiological

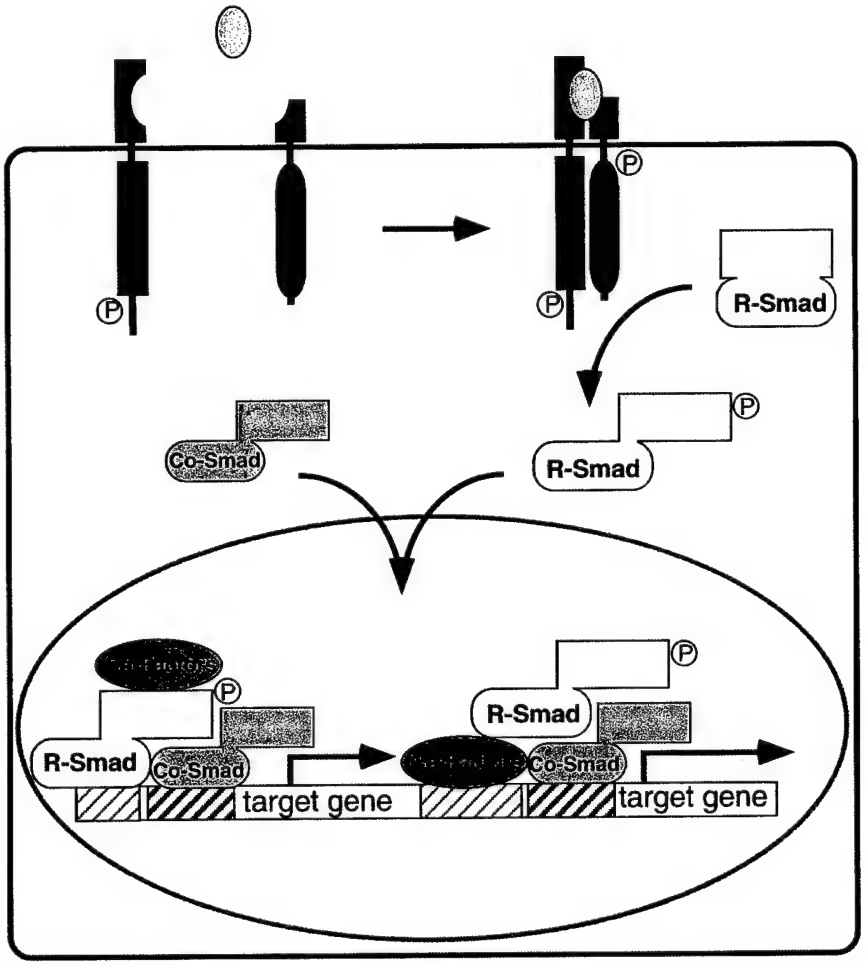

Fig. 3. Model for RSK signal transduction from the membrane to the nucleus. Ligand-bound phosphorylated type I RSKs (1) phosphorylate R-Smads, which associate with the Co-Smads and move into the nucleus. TGFβ-family target genes are induced either by direct binding of Smad complexes to the promoter, or by association of Smad complexes with other DNA binding proteins, such as FAST1. This represents a minimal 'core' TGFβ signaling pathway.

regulators of transcriptional responses was supported by the finding that Smad2 is one member of an activin-induced DNA-binding complex on the promoter of a *Xenopus* immediate–early response gene, *Mix.2* (Chen et al., 1996). The primary DNA binding protein within this activin-dependent complex was shown to be a novel winged-helix transcription factor called Forkhead activin signal transducer (FAST1); Smad4 was also identified as a member of the complex (Chen et al., 1997). Furthermore, TGFβ-induced transcriptional activation was observed in mammalian cells using a reporter gene controlled by the activin responsive element (ARE) from the *Mix.2* promoter, but only in the presence of exogenous FAST1 (Weisberg et al., 1998; Zhou et al., 1998). These findings confirmed that Smads can function as transcriptional regulators within a ligand-induced sequence-specific DNA binding complex.

Proof that Smads function as transcriptional regulators was provided by the observations that the MH1 domain of *Drosophila Mad* can bind directly to specific DNA sequences in the quadrant enhancer of the *vestigial* (*vg*) gene, and that this binding is required for *dpp*-dependent *vg* transcription in vivo (Kim et al., 1997). Subsequently, mammalian Smads 3 and 4 were also shown to bind DNA in vitro. Optimal Smad binding sequences were identified by iterative oligonucleotide selection, and tandemly repeated copies of this sequence [Smad binding element (SBE)] are sufficient to confer TGFβ-responsiveness on a minimal promoter in cell culture (Zawel et al., 1998). The optimal SBE is palindromic (GTCTAGAC) and is found in the promoters of many TGFβ-responsive genes with only minor modifications. Interestingly, Smad2 does not bind to DNA like Smads 3 and 4 due to additional amino acids near the DNA-binding domain present only in Smad2 (Dennler et al., 1999).

Based on these observations and others, discrete functions have been ascribed to the highly conserved Smad MH1 and MH2 domains (Fig. 2). Additionally, the crystal structures of isolated MH1 and MH2 domains have been solved, allowing functional descriptions to be interpreted in light of structural observations (Shi et al., 1997, 1998). The MH1 domain is primarily responsible for binding to Smad-specific DNA-binding elements. The MH2 domain, however, mediates a host of protein–protein interactions, functions as a transcriptional activation motif, and in R-Smads serves as a substrate for type I RSKs.

In summary, Smad proteins mediate RSK-initiated signals from the cell membrane into the nucleus. RSKs are activated via ligand-induced complex formation between type II and type I RSKs. Type II receptor-dependent phosphorylation of the type I receptor at the GS domain activates the type I receptor kinase domain, which then phosphorylates R-Smads on C-terminal serine residues. Phosphorylated R-Smads associate with Co-Smads in the cytoplasm and translocate into the nucleus where transcriptional activation occurs via direct DNA binding, or by association of the Smad complex with other DNA-binding proteins (Fig. 3).

3. Modulation of TGFβ signaling

More recent studies have been directed at understanding intracellular regulation of the Smad pathway, and have focused on how a host of Smad binding proteins modulate TGFβ signaling. In addition, other signaling pathways have now been shown to impinge on the Smad pathway and can either potentiate or inhibit Smad mediated signals. The initially striking simplicity of the core RSK/Smad pathway is quickly giving way to a much more complex view of cellular regulation by TGFβ.

Positive regulators of TGFβ signals include both an upstream accessory protein, known as Smad anchor for receptor activation (SARA), and several downstream effectors that function as either general or tissue-specific transcriptional regulators.

3.1. Positive modulators and effectors

3.1.1. SARA

The first direct cytoplasmic Smad accessory protein to be discovered was called SARA, based on the predicted function of this R-Smad binding protein (Tsukazaki et al., 1998). SARA was identified in a screen for Smad2 MH2 domain interacting proteins, but also appears to bind to Smad3. Conversely, SARA does not bind to Smad 1, nor does it bind to Smad4. Although Smad4 is not a substrate for RSKs and, therefore, may not require a SARA-like partner, the current prediction is that homologous SARA family proteins do exist that bind specifically to R-Smads 1, 5, and 8 and mediate BMP-specific responses. In fact, SARA homologues are present in both *Drosophila* and *C. elegans* genomes, which provides genetic tools for analyzing SARA functions.

In addition to the Smad-binding domain, SARA also contains a sequence motif known as the 'FYVE' domain (Tsukazaki et al., 1998). This is a double zinc finger structure that has been shown to bind phophatidylinositol-3-phosphate and mediate membrane association in yeast and mammalian systems. Direct membrane localization of SARA via the 'FYVE' domain has not been demonstrated, although SARA is present at the membrane and does associate with RSK complexes independent of Smad binding. Further experiments are needed to prove the role of the 'FYVE' domain within SARA.

SARA binds unphosphorylated Smad2, which is released upon ligand-induced phosphorylation and then associates with Smad4 in the cytoplasm; Smad2–Smad4

and Smad2–SARA complexes appear to be mutually exclusive (Tsukazaki et al., 1998). Furthermore, mutations introduced into the 'FYVE' domain of SARA result in different patterns of Smad2 subcellular localization, and also interfere with TGFβ-dependent transcriptional activation. These observations have led to the following model for SARA function. SARA resides at the plasma membrane by virtue of its lipid-binding 'FYVE' domain. Unphosphorylated Smad2 is recognized and concentrated by the SARA Smad-binding domain at the membrane, in close proximity to RSK complexes. RSK activation results in Smad2 phosphorylation, dissociation of SARA from Smad2, and formation of transcriptionally active Smad2–Smad4 heterocomplexes.

The initial discovery and characterization of SARA raises some interesting questions for future investigations. First, how important is SARA's role in TGFβ signaling? Is SARA absolutely required, or is it an enhancer of signaling efficiency in specific cell-types or tissues? Is there a large family of RSK-specific SARA proteins that interact with specific Smads, or can some TGFβ signals be transmitted independently of these adapters? Does SARA function as a scaffolding for assembly of a larger protein complex, and what other protein members might participate? The genetic and biochemical experiments to elucidate these issues are certainly well under way.

3.1.2. FAST proteins

The first nuclear Smad-binding protein was identified as part of an activin-inducible DNA-binding complex in *Xenopus* (Chen et al., 1996). The protein was labeled FAST1, and is a member of the large and diverse winged-helix family of transcription factors. This discovery not only confirmed a biological role for Smads inside the nucleus, but was also the initial observation that the overwhelming majority of direct Smad binding proteins are transcriptional regulators.

As described, FAST1 was identified and cloned based on its activin-induced capacity to bind directly to the promoter of the activin-regulated gene, *Mix.2* (Chen et al., 1996). Subsequently, Smads 2, 3, and 4 were also shown to be present in the complex; Smad2 associates directly with FAST1, whereas Smad4 probably does not bind FAST1 but contributes additional DNA binding specificity and transcriptional activation functions to the complex (Chen et al., 1997; Labbe et al., 1998). It is not known whether FAST1 has any transcriptional activity of its own.

FAST1 homologues have been identified in several species, including mouse and human. Studies using human FAST1 have defined an optimal DNA-binding sequence for these factors (TGT G/T T/G ATT) that is also present in the ARE of the *Xenopus Mix.2* gene. In addition, the importance of adjacent Smad binding sequences was demonstrated in the context of transcriptional activation by the human FAST1 complex (Zhou et al., 1998). Cloning and analysis of a murine homologue, FAST2, indicates that transcriptional regulation by these factors may be more complex. Like the FAST1 complex on the *Mix.2* promoter, FAST2–Smad2 complexes activate transcription from the *goosecoid* (*gsc*) promoter in cultured cells. However, FAST2–Smad3 complexes inhibit transcription from the same promoter (Labbe et al., 1998). This represents the first suggestion that Smad2 and Smad3 may have opposing roles in selected systems.

Whether FAST-like molecules also mediate BMP-initiated signals remains to be determined. To date, no BMP-specific FAST proteins, which would be expected to interact with R-Smads 1, 5, and 8, have been described. However, the Forkhead family of transcription factors is large and homologues are present in *Drosophila* and *C. elegans*. The sheer number of Forkhead family proteins and the limited homology outside the DNA-binding domain make the sequence-based prediction of FAST–Smad combinations quite difficult. Genetic analysis, however, may provide clues as to the identity of any functional FAST–Smad partners.

3.1.3. AP-1 family members

Another group of transcriptional activators with which Smads have now been shown to interact physically are the AP-1 family members, c-Jun and c-Fos. These AP-1 factors have long been suspected to play a role in TGFβ gene regulation based on the requirement of intact AP-1 binding sites in the promoters of several TGFβ-responsive genes, including PAI-1. c-Jun binds directly to amino acids within the variable linker region of Smad3, whereas c-Fos binds within the conserved MH2 domain of Smad3. This ligand-induced complex consisting of Smad3, Smad4, c-Jun, and c-Fos then binds to either overlapping or adjacent AP-1 and Smad binding sequences in target promoters. Transcriptional activation resulting from these cooperative interactions has been demonstrated using the classical TGFβ-responsive promoter PAI-1 (Zhang et al., 1998; Wong et al., 1999).

3.1.4. TFE3

TFE3 is a basic helix–loop–helix transcription factor that has also been shown to cooperate with Smads 3 and 4 to induce PAI-1 transcription in response to TGFβ. All three proteins can bind directly to DNA sequence elements within the PAI-1 promoter, and contribute to maximal TGFβ-dependent transcriptional activation (Hua et al., 1998). Recent data demonstrate that Smad3 binds directly to TFE3, and that this interaction is enhanced when the C-terminal serines of Smad3 are phosphorylated (Hua et al., 1999). The DNA bind-

ing sites employed by TFE3 in cooperation with Smads are distinct from those used by AP-1 family factors, indicating another apparent mechanism for generating specificity at Smad-responsive promoters. Whether TFE3 interacts with AP-1 family members, either functionally or physically, via Smads on this promoter has not been reported.

3.1.5. Vitamin D receptor

Another transcriptional regulator that specifically interacts with Smad3 is the vitamin D receptor (VDR). VDR is a vitamin D-regulated nuclear receptor, similar to the steroid-hormone receptors, that functions with co-activators of the steroid receptor coactivator 1/transcriptional intermediary factor 2 (SRC-1/TIF2) family. This VDR–Smad3 interaction is unique in that VDR appears to bind the MH1 domain of Smad3; most other Smad protein–protein interactions are mediated via the Smad MH2 domain. Additionally, activation of TGFβ signaling results in Smad3-mediated enhancement of VDR-dependent transcription (Yanagi et al., 1999; Yanagisawa et al., 1999). Therefore, Smad3 transcriptional effects are not limited to direct TGFβ target genes, but are also manifest in co-operative enhancement of other signal-regulated pathways.

3.1.6. Polyoma virus enhancer binding protein 2/core binding factor (PEBP2/CBF)

A more recently identified transcription factor that partners with R-Smads to effect changes in gene expression is the PEBP2/CBF, which forms complexes in vivo with both TGFβ and BMP-regulated R-Smads. Interaction with Smad3 requires the MH2 domain, and results in transcriptional activation of the germline Ig Cα promoter. In addition, binding sites for both Smads and PEBP2/CBF are absolutely required for transcriptional regulation. The mechanism of transcriptional control for PEBP2/CBF and Smads may be unique in that PEBP2/CBF appears to interact with all of the R-Smads, and that Smad binding sites appear to play a more direct and necessary role in regulation of the Ig Cα promoter (Hanai et al., 1999).

3.1.7. Olf-1/EBF associated zinc finger (OAZ)

A search for transcription factors that regulate the BMP-responsive gene Xvent-2 in *Xenopus* identified a protein known as OAZ, which was previously identified as a transcription factor involved in the development of rat olfactory epithelium and pre-B lymphocytes. OAZ binds to the BMP-responsive element of the Xvent-2 promoter, and mediates induction of an artificial reporter gene under the control of the BRE (BMP-responsive-element). Furthermore, OAZ can be found in BMP-dependent complexes with both Smad4 and Smad1. The interaction with Smad1 is direct via the MH2 domain, whereas the interaction with Smad 4 is not direct and probably results from the ligand-dependent association of Smad1 with Smad4 (Hata et al., 2000). The identification of a role for OAZ in this context supports the hypothesis that tissue-specific transcription factors may play a prominent role in specifying the cellular effects of Smads, which are ubiquitous TGFβ signal mediators.

3.1.8. MSG1

In addition to acting as TGFβ-regulated transcriptional co-factors, Smads also bind to more ubiquitous co-activators and recruit these accessory proteins to specific promoters. For example, MSG1 is a small nuclear protein with strong transcriptional activity that lacks any identifiable DNA-binding capacity. MSG1 is thought to be important in differentiation and development, although its specific role has not been well-defined. MSG1 was shown by two-hybrid analysis to interact with the MH2 domain of Smad4. TGFβ-dependent transactivation of a GAL4 DNA-binding domain–Smad4 fusion was significantly enhanced by co-expression of MSG 1. This transcriptional enhancement was suppressed by overexpression of the Smad4 MH2 domain, which could sequester MSG1 away from the GAL4–Smad4 fusion (Shioda et al., 1998). Although these results rely on an artificial transcriptional system, they do suggest that Smads can tether more potent transcriptional activators to target genes.

3.1.9. CREB-binding protein (CBP)/p300

More physiologically relevant results supporting this idea are provided by studies of the well-known transcriptional co-activators CBP and p300. These co-activators enhance transactivation by a host of unrelated transcription factors through two different but related mechanisms. First, CBP/p300 brings sequence-specific transcription factor complexes into close proximity to the basal transcriptional machinery, which is required for transactivation. Second, CBP/p300 has intrinsic histone acetylase activity, which modifies chromatin structure and can directly effect the availability of specific promoter sequences to binding proteins.

A number of laboratories have now shown that CBP/p300 binds directly to the phosphorylated MH2 domain of both Smad2 and Smad3. Furthermore, exogenous CBP/p300 augments TGFβ-induced transactivation in a Smad4-dependent manner. Enhancement of Smad transcriptional activity has been shown for a variety of TGFβ-responsive reporter genes in cell culture, including p3TP-Lux-, SBE-Luc-, PAI1-Luc-, and GAL4-based systems. The specificity and requirement for CBP/p300 in these assays was confirmed by showing that the adenoviral protein E1A, an inhibitor of CBP/p300 transcriptional enhancement, blocks the observed increases in Smad-dependent transactivation in the presence of this co-activator (Nishihara et al., 1999). Therefore, Smad proteins provide a critical func-

tion to cell type-specific transcriptional complexes by linking these complexes to the general transcription apparatus through ubiquitous co-activators such as a CBP/p300 (Feng et al., 1998; Janknecht et al., 1998; Pouponnot et al., 1998; Topper et al., 1998).

3.1.10. Homeodomain proteins

One other mechanism of transcriptional activation by Smad1 has recently been reported based on the observation that Smad1 physically associates with one of the homeobox DNA-binding proteins, Hoxc-8. BMP signaling leads to phosphorylation of Smad1, association of Smad1 with Hoxc-8, and transcriptional induction of a Hoxc-8 target gene, osteopontin. However, BMP-induced Smad1–Hoxc-8 complex formation was shown to prevent Hoxc-8 from binding to its target site in the osteopontin promoter. Therefore, Hoxc-8 appears to be a transcriptional repressor, which is inhibited by binding to Smad1 (Shi et al., 1999). This represents the first evidence that Smads may allow for transcriptional activation indirectly by physically interfering with functional transcriptional repressors.

The idea that TGFβ-family signals may de-repress selected genes via Smad-mediated events is also suggested by studies of the *daf-3* Smad in *C. elegans*. Genetic analyses clearly demonstrate that *daf-3* is antagonized by RSKs and other Smads in *C. elegans*. In the absence of TGFβ signaling, *daf-3* causes worms to enter an alternative developmental stage known as dauer; accordingly, RSK and Smad mutants undergo *daf-3*-induced dauer formation [Patterson et al., 1997; reviewed in Patterson and Padgett (2000)]. An intact activated TGFβ signaling pathway, however, blocks *daf-3*-induced dauer arrest. Furthermore, *daf-3* has been shown to bind directly to DNA and to repress transcription from a pharynx-specific promoter element. In *daf-3* mutants, repression is relieved and pharynx-specific reporter expression is restored (Thatcher et al., 1999). Whether there are additional protein partners in a *daf-3* complex is not yet known.

However, there is now clear precedent for future investigations of transcriptional de-repression mediated through TGFβ-initiated Smad-dependent processes. There are also several other transcription factors that may cooperate with Smads to induce gene transcription, but which have not been shown to interact physically with Smads. For example, SP1-family transcription factors have been implicated in TGFβ signal mediation by virtue of the requirement of intact SP1-binding sites in the p15 and p21 CKI promoters for TGFβ-responsiveness (Datto et al., 1995; Moustakas and Kardassis, 1998). Similarly, the *Drosophila* protein SCHNURRI plays some role in *dpp* signaling and gene regulation, but confirmation that it cooperates with Smads has been elusive (Arora et al., 1995; Greider et al., 1995).

These many examples clearly demonstrate that protein partners for TGFβ-regulated Smad proteins are indispensable for appropriate activation and modulation of Smad target genes. Furthermore, it appears that Smads regulate transcriptional responses in a number of related ways. Smads can bind to other sequence-specific DNA-binding proteins and provide a transactivation function. Smads can bind DNA at defined target sequences and tether other transcriptional co-activators to these promoters. Finally, Smads may be able to activate transcription by either direct or indirect de-repression of silenced genes. In any case, Smad proteins are clearly very versatile TGFβ-sensitive transcriptional modulators.

3.2. Negative modulators and inhibitors

In addition to Smad co-factors that positively regulate or enhance transcriptional outputs, a number of other proteins have been discovered that attenuate TGFβ signaling by interfering with Smad functions. These negatively acting Smad partners are required to prevent the inappropriate activation of TGFβ pathways, and to turn off the pathway following normal activation.

Although there are many ways by which TGFβ signals are inhibited, including the extracellular sequestration of ligands by proteins such as follistatin, chordin, and noggin, and the intracellular regulation of RSK activation by FKBP12, we will focus primarily on direct Smad regulators. The molecules highlighted here function at different levels within the signaling pathway: Anti-Smads competitively inhibit Smad functions, transcriptional repressors block activation of Smad target genes in the nucleus, and ubiquitin-dependent proteases degrade Smads and attenuate TGFβ signaling. Interestingly, the most logical of potential Smad inhibitors based on activation by RSK phosphorylation — a phosphatase — has not yet been discovered.

3.2.1. Anti-Smads

The first Smad inhibitors to be described were another class of divergent Smad proteins known simply as inhibitory Smads or Anti-Smads. These Smads contain divergent MH1 domains but share the conserved MH2 domain with R-Smads and Co-Smads; however, they lack C-terminal phosphorylation sites. Smads 6 and 7, along with a *Drosophila* homologue, *Daughters against dpp* (*Dad*), function as negative feedback inhibitors of TGFβ-family signaling. Anti-Smads are transcriptionally induced by the ligands that they ultimately inhibit, as well as by shear stress in vascular endothelium (Nakao et al., 1997a; Topper et al., 1997; Tsuneizumi et al., 1997; Nakayama et al., 1998).

Two mechanisms have been proposed for how Anti-Smads exert their inhibitory function. Each of the Anti-Smads can bind to RSK complexes and prevent phosphorylation of R-Smads (Hayashi et al., 1997; Inamura

et al., 1997; Nakao et al., 1997a; Tsuneizumi et al., 1997). This, in turn, prevents Smad heteromer formation and nuclear accumulation and explains the non-specific inhibition observed when Anti-Smads are overexpressed in cell culture. An alternate mechanism that accounts for the specific inhibition of BMP signaling by Smad6 is based on the observation that Smad6, when expressed at lower levels, does not prevent phosphorylation of Smad1, but instead competes with Smad4 for binding to phosphorylated Smad1 (Hata et al., 1998). Additional analyses of endogenous Anti-Smads will be required to refine these models.

3.2.2. Co-repressors

Another mechanism by which Smad transcriptional activity can be inhibited is via direct binding to transcriptional co-repressors, or binding to intermediary proteins that recruit such repressors. Two prominent examples of this type of Smad inhibition have been described. First, another homeodomain protein, 5′ TG 3′ interacting factor (TGIF), was identified as a Smad2-binding protein using the yeast two-hybrid system. Although the function of TGIF is not well understood, this study showed that it can function as a transcriptional repressor at Smad-responsive promoters. Furthermore, inhibitors of histone deacetylases (HDACs) were shown to reduce the TGIF-dependent repression, suggesting that HDACs may be recruited to Smad-responsive promoters via interaction with TGIF–Smad complexes (Wotton et al., 1999). However, Neither a specific HDAC protein nor direct interaction with TGIF was conclusively demonstrated.

Because Smads have been shown to enhance transcription by association with histone acetylases such as CBP/p300, the possibility that repression of Smad-mediated transactivation occurs through histone deacetylation is rational. The role of histone acetylation in transcriptional regulation has been well-documented; acetylation of core histones disrupts the packing structure of DNA thus increasing accessibility to DNA-binding proteins, whereas deacetylation allows orderly packing and excludes transcription factors (Lee et al., 1993; Wolffe, 1996). Confirmation that HDACs play a role in repressing Smad-regulated promoters has been provided by the observation that Smads interact directly with the related HDAC-binding oncoproteins Ski and Sno. Both Ski and Sno have been found in human tumor cell lines and both can transform cells in culture; this implies that these proteins function oppositely to Smads. TGFβ-dependent interaction of Smads 2, 3, and 4 with Ski and Sno results in transcriptional repression of several different Smad-responsive promoters. Repression was shown to be dependent on the presence of the nuclear hormone receptor co-repressor (N-CoR) within the Smad–Sno/Ski complex, and the N-CoR-associated HDAC. In these studies, the presence of HDAC1 in the Smad complex was demonstrated. In addition, exogenous Ski was shown to reduce Smad-associated histone acetylase activity (presumably due to CBP/p300) in a dose-dependent manner (Akiyoshi et al., 1999; Luo et al., 1999; Stroschein et al., 1999; Sun et al., 1999a,b). Finally, both TGIF and Ski compete with CBP/p300 for binding to Smad complexes (Akiyoshi et al., 1999; Wotton et al., 1999). Together, these observations demonstrate that Smad transcriptional activity can be inhibited by physical association with co-repressors that tether HDACs to Smad-responsive promoters.

3.2.3. Smad ubiquitination regulatory factor 1 (Smurf1)

The large number of potential Smad-binding partners implies that the amount of Smad protein available inside the cell is a key determinant of ultimate Smad function. Consistent with this hypothesis, the directed proteolysis of BMP-responsive R-Smads has been reported. Smads 1 and 5 were shown to interact with a novel ubiquitin ligase discovered as a Smad1-binding protein using two-hybrid screening. Smurf1 shares sequence homology with the Hect subclass of E3 ubiquitin ligases, and specifically targets Smads 1 and 5 for ubiquitination and subsequent degradation. Smurf1 function was confirmed in *Xenopus* embryos, where ectopic expression inhibits Smad1-induced ventralization (Zhu et al., 1999). Ubiquitination and degradation of Smad1 was independent of BMP receptor activation, leaving open the question of how Smad proteolysis is regulated in vivo.

Ubiquitin-dependent degradation of nuclear Smad2 has also been recently demonstrated (Lo and Massagué, 1999). In this case, the nuclear localization of Smad2 appears to be the key determinant of ubiquitination and degradation, rather than phosphorylation per se. However, because RSK-mediated phosphorylation precedes nuclear accumulation of Smads in vivo, it is reasonable to hypothesize that TGFβ signals are attenuated in part by degradation of activated R-Smads.

3.2.4. Calmodulin

Calmodulin, the primary mediator of calcium signaling, has also been shown to interact physically with R-Smads and Co-Smads in vitro, and to inhibit TGFβ signaling in cultured cells. Calmodulin binds two different amphiphilic α-helices in the MH1 domain of R-Smads in a strictly calcium-dependent manner; calmodulin is one of only two proteins that interact with Smads through the MH1 domain. Exogenous calmodulin also inhibits Smad-mediated transactivation from multiple TGFβ-responsive promoters. Furthermore, inhibition of calmodulin function allows increased expression of Smad-dependent reporter genes (Zimmerman et al., 1998). Because calmodulin interacts with a large number of other proteins, and because calcium signaling regulates a diverse set of cellular

processes, more work is required to determine the physiological significance of these observations. However, the roles of other signaling pathways in modulating TGFβ responses are now also being elucidated.

4. Cross-talk with other signaling pathways

Signaling by interferon-γ is mediated by cytokine receptors that activate JAK tyrosine kinases and, subsequently, STAT proteins. Interferon-γ inhibits TGFβ signaling by the direct STAT-mediated transcriptional induction of Smad7. Smad7, of course, is an Anti-Smad that prevents R-Smad phosphorylation, R-Smad and Co-Smad complex formation, and nuclear accumulation of the complexes (Ulloa et al., 1999). Similarly, the inhibition of TGFβ signaling by TNFα has also recently been shown to rely on Smad7 induction, in this case by NF-κB/Rel A (Bitzer et al., 2000).

Conversely, cooperation between STAT3 and Smad7 in primary fetal neural progenitor cells has been demonstrated (Nakashima et al., 1999). In these cells, leukemia inhibitory factor (LIF) activates STAT3, which synergizes with BMP2-activated Smad1 to effect differentiation into astrocytes. This apparently results from the physical association of both STAT3 and Smad1 with p300 (described previously) and subsequent transcriptional modulation. Therefore, the relationship between Smads and STATs is complex and the ultimate effects of simultaneously activating these factors may be highly cell-type specific.

In a similar way, the classical MAP kinase pathway has been implicated in both positive and negative regulation of TGFβ signaling at the level of Smad function. Positive regulation of Smads results from EGF and HGF receptor activation, which causes phosphorylation of Smad2 at sites independent of the C-terminal serines targeted by RSKs. EGF- and HGF-dependent phosphorylation of Smad2 results in activation of Smad2-responsive reporter genes, suggesting that MAP kinases may be able to potentiate Smad signaling in addition to RSKs (de Caestecker et al., 1998; Brown et al., 1999). However, EGF and HGF stimulation of MAP kinases has also been shown to inhibit Smad signaling. Phosphorylation at consensus MAP kinase sites within the variable linker region of R-Smads prevents nuclear accumulation of Smad complexes, and transcriptional induction of Smad-dependent reporter genes (Kretzschmar et al., 1997a, 1999). The reason for the striking discrepancy between these reports is not clear. Could these opposing effects be the result of cell-type specific differences, or could EGF and HGF stimulation differentially modify Smad-binding proteins to change signal outputs? There are several complex possibilities, but only more work will distinguish between them.

What is clear is that Smads integrate a variety of signal inputs, and interact with a host of cellular proteins to modify transcriptional responses.

5. Conclusion

In summary, Smad proteins mediate TGFβ-initiated RSK signals from the plasma membrane into the nucleus. Activation of RSKs occurs through ligand binding and complex formation between type II and type I receptor subunits. Type II receptor-dependent phosphorylation of the type I receptor at the GS domain activates the type I receptor kinase, which phosphorylates an R-Smad on C-terminal serines. The phosphorylated R-Smad associates with a Co-Smad in the cytoplasm and then moves into the nucleus, where transcriptional regulation occurs via direct DNA binding by the Smad complex, or by association of the Smad complex with other DNA-binding proteins (Fig. 3).

Smad functions can be enhanced or inhibited by a large variety of partner binding proteins. DNA-binding proteins, transcription factors, and transcriptional co-activators all participate with Smads in transactivation. Conversely, Anti-Smads, transcriptional repressors, and elements of the protein degradation machinery all antagonize Smad functions. The core TGFβ signaling pathway is certainly a simplified description of this critically important regulatory network.

Future investigations will focus on clarifying many of the issues discussed here, and on several other interesting questions. Learning more about the genes regulated by TGFβs will be a necessary step in understanding how these factors modulate such a diverse range of biological processes. In addition, clarifying the role of a limited set of receptors for a larger group of ligands will be important. Another open question is whether the Smads are the only TGFβ signal mediators. The sheer diversity of ligand functions implies that other mediators may be present, and there is suggestive evidence that other mediators exist. The models and tools are now available to address these issues, and to generate the next set of interesting questions.

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Mini-review

Genetic approaches to TGFβ signaling pathways

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1. Introduction

At the turn of the century, when Thomas Hunt Morgan started using the fruitfly, *Drosophila melanogaster*, for genetic experiments, the degree of conservation of many basic cellular processes between diverse metazoan species had not yet been realized. Later, as the various biological disciplines developed, it became clear that this was indeed the case. This insight spurred Sydney Brenner in the late 1960s to develop the nematode, *Caenorhabditis elegans*, into a model organism for studying nervous system structure and development as an avenue to understanding these processes in higher organisms [1]. Even when Nüsslein-Volhard and Wieschaus [2] did their pioneering work in identifying patterning genes in *Drosophila* in the late 1970s, it was not yet widely accepted that similar genes would be used to pattern other metazoa. We now know that the overwhelming majority of cellular and developmental processes are highly conserved, and that *C. elegans* and *Drosophila* are excellent model systems to study these processes.

The widespread use of *C. elegans* and *Drosophila* is attributable to the fact that both are multicellular organisms which can be manipulated with a number of sophisticated molecular and genetic tools, making the identification and characterization of mutant loci a practical option. Further, the genome sequencing projects in the two organisms have proved to be a valuable asset. While over 99% of the *C. elegans* genome has been sequenced and published [3], the *Drosophila* genome project is expected to reach completion shortly. Genome sequencing provides easier access to gene identification, and has reinforced the finding that many genes and pathways are evolutionarily conserved.

In this review, we provide a summary of the genetic analysis of TGFβ signal transduction, as well as its role in various human diseases and mouse models. We also use discoveries in the TGFβ pathway as an example to highlight some of the techniques used in the invertebrate world of *C. elegans* and *Drosophila* to further our understanding of this, and other, signaling systems. The roles of such techniques in elucidating diverse signaling pathways, as well as pathways of human disease genes, will become more important as the information from the genome projects increases and as the development of genetic tools to analyze them becomes more powerful. Given the conservation of signaling mechanisms, there will be increasing synergy between studies in invertebrates and vertebrates in future years for solving different cellular pathways.

2. Genetic analysis of TGFβ signaling

2.1. Superfamily ligands

TGFβ superfamily members are secreted growth factors that possess several important functions in cell proliferation, differentiation, and adhesion (reviewed in [4]). The TGFβ family is divided into three primary groups, namely the TGFβs, activins, and bone morphogenetic proteins (BMPs), each comprised of ligands with specific roles in development and homeostasis.

The *Drosophila* gene *decapentaplegic* (*dpp*) was identified as a TGFβ member [5] soon after the first vertebrate members of the superfamily were cloned. *dpp* was known to have several critical functions in *Drosophila* development, and had been genetically well

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characterized [6–8]. It was also shown that vertebrate BMP4 and *Drosophila dpp* could functionally substitute for each other, providing important evidence for the conservation of structure and function within the superfamily [9,10]. These results gave credence to the use of the *Drosophila* system for analysis of the pathway. In recent years, several other homologs have been identified from both *Drosophila* and *C. elegans* [11–15] and as is discussed below, the study of these invertebrate ligands has been crucial in providing important breakthroughs in understanding TGFβ signaling in other organisms.

The recent innovation of some genetic tricks in *Drosophila* have permitted the testing of various models of ligand function. These innovations include the ability to induce clones of cells that are either mutant for a particular gene [16], or over-express it in tissues where it is not normally expressed, using the FLP/FRT and the UAS/GAL4 techniques [17,18]. Similar tools have been used in a series of elegant experiments to demonstrate that *dpp* and *wingless* function as classical morphogens [19–21].

2.2. Receptors

TGFβ superfamily ligands have been shown to signal via serine/threonine (S/T) kinase receptors (reviewed in [22]). These receptors were first cloned from vertebrates [23], and were later shown to belong to two related groups — the type I and II groups. It was observed that these genes were homologous, to *daf-1*, a previously cloned orphan S/T kinase receptor in *C. elegans* [24]. *daf-1* had been isolated as a gene that functions to control entry into the dauer stage, wherein the animals become resistant to harsh environmental conditions. A type II receptor gene, *daf-4*, has since been cloned from *C. elegans*, and shown to function with the type I receptor, *daf-1*, in the dauer pathway [25]. Curiously however, *daf-4* mutants were observed to have phenotypes that were not displayed by *daf-1* mutants, leading to the hypothesis that *daf-4* may function in two distinct signaling pathways to control different patterning events [26]. Such genetic analyses are a powerful tool in studying signal transduction, and can be of enormous help in identifying partner molecules. The hypothesis stated above, based on the examination of mutant phenotypes, was borne out by subsequent discoveries [26], and is examined in more detail below.

It has been demonstrated that the TGFβ ligands may signal as either homo- or hetero-dimers, by binding both type I and type II receptors. The type II receptor phosphorylates and activates the type I receptor, which then transduces the signal to downstream target molecules [27]. In *Drosophila*, the *dpp* receptor genes *punt* (type II), *thick veins*, and *saxophone* (both type I) have been cloned and shown to be critical for *dpp* signaling [20,28–32]. As these genes function together to pattern the same morphological structures, animals mutant for any one of them display similar phenotypic defects. The *dpp* pathway provides a good case study of another kind of genetic trick — looking for loci that mutate to similar phenotypes, and then cloning the genes responsible. *Schnurri*, for example, was identified by first scrutinizing various loci for ones that mutated to yield a ventralized embryo, a characteristic of *dpp* alleles, and then cloning the gene molecularly [33,34].

2.3. Smads

After the characterization of the ligands and receptors, further elucidation of the TGFβ pathway was stalled. Various molecular and biochemical tools, such as two-hybrid and immunoprecipitation experiments, failed to yield any convincing downstream signal transducers. The breakthrough in this area was facilitated by the genetic analysis of TGFβ signaling in both *Drosophila* and *C. elegans*. Screens designed to identify mutants that enhanced the morphological defects of *dpp* alleles [35,36], resulted in the isolation of *Mothers against dpp* (*Mad*), a gene later shown to encode a novel protein [36]. Similar studies in *C. elegans* resulted in the isolation of three genes, *sma-2, sma-3*, and *sma-4*, with mutant phenotypes similar to alleles of the type II receptor gene, *daf-4*. Upon cloning, all three loci proved to encode molecules homologous to *Drosophila Mad* [26]. The identification of these four related genes, now called the Smad family [37], as conserved TGFβ signal transducers from two different invertebrate organisms, led to the cloning and biochemical characterization of several vertebrate homologs. It is now clear that the Smads form an integral part of the transduction process (reviewed in [38,39]).

It has been shown that the Smads are phosphorylated by the activated ligand-receptor complex. They then translocate to the nucleus, where they regulate the expression of target genes in conjunction with specific transcription factors. Smad4, the first vertebrate Smad to be functionally characterized, was shown to be mutant in several different tumorigenic tissues [40–43]. Without the invertebrate data linking these genes to TFGβ signaling, the role of Smad4 in cancer would have been unclear.

3. TGFβ signaling defects in mouse models

Targeted disruptions of the vertebrate Smads over the past few years have also yielded insights into the functions of these molecules in TGFβ signal transduc-

Table 1
Loss-of-function phenotypes associated with various TGFβ pathway components

Organism	Mutated or deleted TGFβ component	Defect
Human	MIS	Müllerian Duct Syndrome [58,59,71]
	MIS type II receptor	
	CDMP-1 (GDF-5)	Brachydactyly [72], abnormalities in limb bone development [73,74]
	TβRII	Colon, head and neck cancers [54,75]
	TβRI	Metastatic breast cancers [76], colon, gastric and prostatic cancers, AIDS-related kaposis sarcoma (reviewed in [54]), chronic lymphocytic leukemia [77]
	ALKI	Human hereditary hemorrhagic telangiectasia (HHT) [55–57]
	Endoglin	
	Smad2	Colorectal and lung cancers [61,62]
	Smad4	Breast, colorectal, esophageal, head and neck, lung, ovarian, pancreatic, prostatic, and gastric cancers [40–43,78,79], juvenile polyposis [80], biliary duct carcinoma [81]
Mouse	TGFβ1	Abnormal modulation of immune system [82], multifocal inflammatory lesions [83]
	BMP2	Abnormal cardiac development, malformation of amnion/chorion [84]
	BMP4	Defects in gastrulation and mesoderm formation [85]
	BMP5	Defects in skeletal and soft tissue development [86,87]
	BMP7	Abnormalities seen in skeletal, kidney and eye development [88,89]
	Smad2	Embryonic lethal (E7.5–E10.5), defective mesoderm formation [44–46]
	Smad3	Defective immune function [48], develop colorectal cancers [47,49]
	Smad4	Embryonic lethal (die before E7.5), fail to initiate gastrulation, in combination with APC mutations, aids in the progression of colorectal tumors [50,51,90]
	Smad5	Embryonic lethal (E9.5–11.5), defects in heart development [53]
Drosophila	Dpp pathway	Immaginal disc and dorsal-ventral patterning defects [6–8]
	Activin pathway	Defects in cell growth and proliferation [91,92]
C. elegans	Sma/Mab pathway	Small body size, male tail ray fusions, crumpled spicules [26,93]
	Dauer pathway	Defective in entering or exiting a developmentally arrested state [94]

tion (Table 1), and have allowed the testing of the models derived through in vitro studies.

Homozygous *Smad2* mutants die as early embryos due to defective mesoderm formation and other defects [44–46]. This is a more severe phenotype than its putative upstream activators, such as TGFβs and activins. In contrast, *Smad3* mutants are normal at birth and go on to become slightly smaller adults than their litter-mates. They do, however, exhibit a compromised immune system, and die from immune-related complications between one and eight months of age [47,48]. Many Smad3 deficient mice also develop metastatic colorectal carcinoma [49]. Biochemically, Smad2 and Smad3 behave in very similar ways, except for the observation that Smad2 is unable to bind DNA, while Smad3 does. Now these in vivo data from mouse knock-outs support the fact that they may have different functions.

Homozygous *Smad4* mutant mice have the most severe phenotypes of all Smad knock-out mice, a logical result given the evidence pointing to its central role as a Co-Smad in all TGFβ superfamily signaling. They fail to initiate gastrulation and die as early embryos [50,51]. They show defective mesoderm formation and growth retardation, which have been observed in *BMP4*- and *activin*-deficient mice. They also show visceral endoderm abnormalities. The defects in mesoderm formation can be rescued by aggregating the mutant embryos with wild-type tetraploid cells, which can only contribute to the development of extraembryonic tissue [50]. This suggests that Smad4 may not have a function in BMP signaling in early embryonic stages, or that another BMP-specific homolog of Smad4 has not yet been identified. These data are in contrast to the biochemical studies that have demonstrated the requirement for Smad4 in BMP, TGFβ, and activin signaling. However, the recent finding that in *Xenopus*, two forms of Smad4 exist, each with its own unique characteristics [52], supports the idea that the mouse may have multiple Smad4 genes with different functionalities.

Mutations in *Smad5* lead to lethality in late embryogenesis [53]. These embryos undergo normal gastrulation, and begin organogenesis before dying. The loss of Smad5, which has been biochemically determined to function in the BMP pathway, leads to phenotypes that are less severe than those of mutant BMP ligands or receptors. Interestingly, Smad 5 homozygotes show defects in heart development, much like BMP-2 deficient mice [53]. It has been shown in vitro that

Smad5 may act with Smad1 and Smad8 in BMP signaling, and this overlap of function may explain the reduced severity of the mutations.

4. TGFβ mutations in human diseases

Given the varied and important functions of TGFβ pathways in the development and homeostasis of different tissues, it is not surprising that many connections have been found to link mutations in these components to the formation of human diseases (Table 1).

One of the first connections between TGFβ signaling and cancer was drawn from the identification of mutations of the TGFβ type II receptor. These mutations are found in a significant percentage of hereditary or somatic forms of nonpolyposis colorectal cancer with microsatellite instability [54]. The mutations make cells refractive to TGFβ signals, and lead to an enhancement of the tumorigenic state of cells. This type of cancer accounts for about 10% of all colorectal cancers.

More recently, mutations in *endoglin*, a TGFβ accessory protein, or in *ALK-I*, a type I receptor, have been shown to lead to hereditary hemorrhagic telangiectasia (HHT), a disease in which the vascular structure develops abnormally, and where a tendency is seen for the nasal mucosa to rupture frequently [55–57]. Type III receptors are thought to only help present ligand to the receptor, and are thought of as being critical components of the pathway. In vitro experiments have shown that endoglin can only weakly bind any of the known ligands, suggesting that this disease state is caused by the disruption of signaling of an as-yet unidentified ligand.

Müllerian Inhibiting Substance (MIS), a more distant member of the TGFβ superfamily, is required for the normal regression of the Müllerian duct during male development. In instances where MIS is disrupted, this regression fails to occur, and leads to pseudo-hermaphroditism in males. The disease, known as the Müllerian duct syndrome [58,59], can be caused in both humans and mice by mutations in the ligand or its receptor [60].

The intensive research into Smads in the recent past has uncovered a number of connections with various cancers. Smad4, previously called *DPC4* (deleted in pancreatic carcinoma, locus 4), is found to be mutant in over 50% of all pancreatic carcinoma [40]. In addition, it is also mutated in a significant percentage of colorectal carcinoma, and less frequently in other cancers such as prostatic, ovarian, and breast [41–43]. This difference in the frequency of Smad4 mutations in various cancers may reflect a difference in the role of TGFβ signaling in the homeostasis of these tissues. Smad2 has also been found to be mutated in a small number of colorectal cancers [61,62]. From these results, some interesting observations may be made, such as the fact that Smad4 appears to be the only Smad mutated in an overwhelming majority of cases. This may reflect the key nature of the role in TGFβ signaling, or may reflect some aspect of TGFβ function in tissues. The answers to such questions should lead to other exciting observations as well.

5. Strategies in invertebrate TGFβ signaling

5.1. Genetic screens

As described above, screens for novel TGFβ pathway components, based on the mutant phenotypes observed for ligands or receptors in *Drosophila* and *C. elegans*, have resulted in a rapid elucidation of not only these invertebrate developmental signals, but also of their human counterparts. In the context of TGFβ signaling, screens have also been performed in *C. elegans* and *Drosophila*, for genes that mutate to a similar phenotype as known components, and it is expected that the cloning of these genes will further define the mechanism of signaling by identifying additional components. Similar strategies can be adopted for almost any signaling system. For example, Forrester et al. [63] describe screens for genes involved in regulating the migration of developing neurons, wherein they are able to score for those morphological traits that are frequently associated with neuronal migration defects.

In addition to the general strategies outlined above, model systems make available a range of phenotypes and elegant genetic tricks that can be benefited from. For example, over-expression of the *dpp* receptor *thick veins*, causes the mis-patterning of wing or eye tissues, and these phenotypes may be enhanced or ameliorated by removing one copy of a gene that normally functions in the pathway and is necessary for the activity of *thick veins*. These 'modifier' screens have also been used to elucidate other signaling systems, such as the MAP kinase pathways (e.g. see [64]).

One key feature of *C. elegans* biology is that the lineage of each cell in the adult worm is completely known. Every hermaphrodite worm is born with 588 cells that divide to give adults with 959 cells. It is possible to trace the 'family tree' of each of these cells in the adult, and to determine the exact nature of the defect in worms with morphological defects, therefore providing the opportunity to study complex phenotypes at a very basic level (e.g. see [65]). In addition, over- or mis-expression of a gene may cause subtle phenotypic defects that can be used to study aspects of the gene that may not otherwise have been possible. These phenotypes can then be used for the further isolation of partner molecules.

The complete sequencing of the *C. elegans* genome has emphasized the need for the development of new tools for reverse genetics. The quick generation time of the nematode adds to its attraction as a good model organism, and has made the development of these tools simple to accomplish. Two ideas that show great promise are discussed below. First RNA interference (RNA-i) can be used for transient "knock outs", and secondly, reverse genetics using various PCR-based assays can be performed to identify mutations in the gene of interest. One of the popular techniques for reverse genetics utilizes transposable elements [66], and another involves the use of chemical mutagenesis ([67] and G. Moulder, R. Barstead, C. Johnson, personal communications).

5.2. RNA interference (RNA-i)

Although the sequence of the *C. elegans* genome is available, this effort has only yielded the molecular identities of the many loci in the genome. The functions of the proteins encoded by each of those loci, however, still need to be determined. A recently developed technique, referred to as RNA interference (RNA-i), has proven to be an invaluable tool in this regard (Fig. 1). Double-stranded RNA from the coding region of a gene injected into an adult *C. elegans* gonad interferes with endogenous gene activity causing a mutant phenotype specific for that particular RNA [68]. Previously, single stranded antisense RNA has been used, with limited success, to interfere with gene activity. Extremely high concentrations of RNA are needed to reveal even a modest phenotype. Fire et al. [68] have shown that injection of double-stranded RNA at much lower concentrations produces a more severe phenotype, similar to phenotypes observed in animals with null mutations, indicating specificity of interference. The actual mechanism by which double-stranded RNA interferes with gene activity is unclear. This powerful technique has been successfully used to identify the function of a variety of genes in *C. elegans*. It has recently been successfully extended to *Drosophila* as well, where double-stranded RNA was injected into syncitial blastoderm embryos to show that the *frizzled* and *frizzled 2* genes are a part of the *wingless* pathway [69], and to show that the MyoD homolog, *nautilus*, was essential for muscle formation [70]. In addition to a simple demonstration of their role in the pathway, it was shown that the patterning defects were not caused by the injection of either RNA alone, but required the injection of both RNAs together. Such a hypothesis would previously have required the generation of mutants in both genes to be proven. The extension of this technique to other organisms will further increase the value and acceptability of this method for studying gene structure and function.

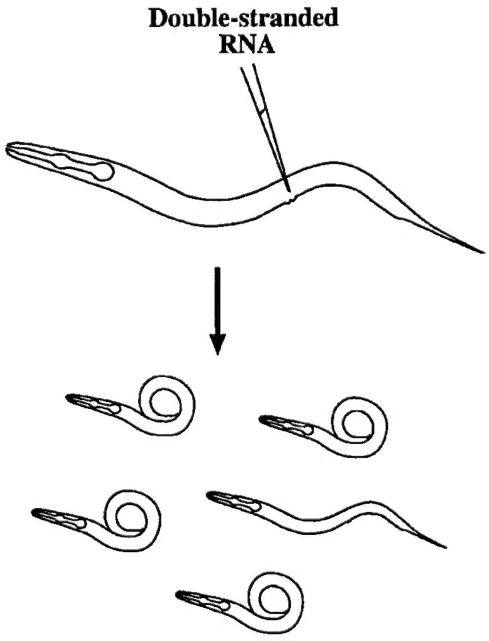

Fig. 1. RNA-interference (RNA-i): a technique used to determine the mutant phenotype of a gene. Double-stranded RNA, comprising both sense and antisense strands, is injected into animals. Interference with wild-type gene function causes a mimicking of the mutant phenotype of the gene. This technique, pioneered in *C. elegans*, is applicable to some other organisms.

5.3. Reverse genetics in C. elegans

Once a gene of interest has been identified, it is necessary to obtain mutants to further characterize its function. One method of generating mutants that has become popular recently, is based upon the use of ethyl methanesulphonate (EMS) or trimethylpsoralen (TMP), chemicals that cause random deletions within the genome at low frequencies. The worms are first mutagenized, then distributed onto agarose plates with an appropriate food supply and grown until the population is dense. Next, DNA is harvested from a subset of each population and analyzed by PCR. PCR is performed using nested primers 1–3 Kb apart. Animals in which the gene of interest has incurred a deletion will yield PCR products smaller than those observed from wild-type worms (Fig. 2). PCR-positive reactions identify the original plate, from which the worms are recovered and re-plated at a lower density. This process is repeated until a single mutant worm is identified. Usually, 1×10^6 mutagenized genomes result in the identification of targeted deletions approximately 50% of the time.

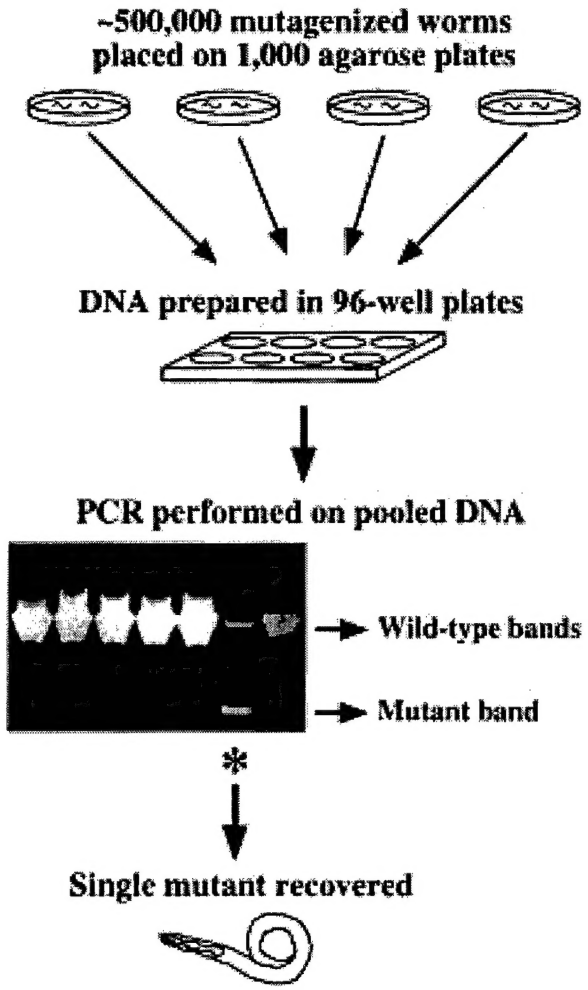

Fig. 2. A novel approach to reverse genetics. A mutagen is used to generate deletions in the genome, and a PCR-based strategy is used to screen for, and isolate, the worm carrying a deletion in the gene of interest.

6. Conclusions

The genetic analysis of TGFβ and other pathways in invertebrates provides an effective way to quickly identify partner molecules, and characterize their roles in signaling. Genetics in mice and zebrafish complement these studies, while biochemical studies in cell culture add functionality.

In summary, screens and related genetic strategies have been very rewarding in the elucidation of the TGFβ signal transduction pathway. These strategies can also be used to study genes involved in a variety of important processes, such as axonal migration and differentiation, neuronal degeneration, apoptosis, and cell cycle. Invertebrate model systems can also be used to study important human disease genes. For example genes involved in Huntington's or Alzheimer's diseases may be studied by expressing wild-type or mutant forms of the protein to produce phenotypes that could then be used in modifier screens. The validity of such approaches has gained widespread acceptance with several biotechnology companies and individual laboratories using genetics in invertebrate organisms to gain insights into the functions of human genes.

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